BETA-GALACTOSIDASE PRODUCTION FROM *Streptococcus thermophilus* ISOLATED FROM MILK YOGHURT

A Dissertation submitted to

THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY, CHENNAI.

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MASTER OF PHARMACY

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Submitted By

Reg. No: 26063826

Under the supervision of

Mr. K. Kamalakannan, M. Pharm.

Department of Pharmaceutical Bio-technology

Nandha College of Pharmacy

Erode – 638 052, (TAMILNADU)

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CERTIFICATE

This is to certify that the work embodied in this thesis entitled, "**BETA-GALACTOSIDASE PRODUCTION FROM *Streptococcus thermophilus* ISOLATED FROM MILK YOGHURT"** submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai was carried out by S. Princely E. Gnanakani, in the Department of Pharmaceutical Biotechnology, **Nandha College of Pharmacy, Erode-52** for the partial fulfillment for the award of degree of Master of Pharmacy in Pharmaceutical Biotechnology under my direct supervision.

This work is original and has not been previously formed the basic for the award of other degree, diploma, associateship, fellowship or any other similar title and the dissertation represent entirely an independent work on the part of the Candidate.

Mr. K. KAMALAKANNAN, M. Pharm.,
Assistant Professor,
Department of Pharmaceutical Biotechnology,
Nandha College of Pharmacy,
Erode-52.

DECLARATION

The work presented in this thesis entitled **"BETA-GALACTOSIDASE PRODUCTION FROM *Streptococcus thermophilus* ISOLATED FROM MILK YOGHURT"** was carried out by me in the Department of Pharmaceutical Biotechnology, Nandha College of Pharmacy, Erode under direct supervision of **Mr. K. Kamalakannan, M. Pharm.**, Asst. Professor, Department of Pharmaceutical Biotechnology, **Nandha College of Pharmacy, Erode-52.** This work is original and has not been submitted in part or full for the award of other degree or diploma of any other university.

Place: S. Princely E. Gnanakani

Date:

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ABBREVIATIONS

%	:	Percentage
β	:	Beta
±	:	Plus or minus
µ moles/ml	:	micro moles per milli liter
°C	:	Degree Celsius
µg/ml	:	micro gram per milli liter
µl	:	micro liter
µmol	:	micro mole
β-Gal	:	Beta-Galactosidase
BSA	:	Bovine serum albumin
EDTA	:	Ethidine diamine tetra acetate
gm	:	gram
hr	:	hour
Lc	:	*Lactococcus*
Lb	:	*Lactobacillus*
M	:	molar
mg	:	milli gram
mg/ml	:	milli gram per milli liter
min	:	minute
mM	:	milli Molar
n moles/ml	:	nano moles per milli liter
nm	:	nano meter
ONP	:	Ortho-nitrophenol
ONPG	:	Ortho nitrophenyl β - D - Galactopyranosidase
rpm	:	revolution per minute
S	:	saturation
S. thermophilus	:	*Streptococcus thermophilus*
[S]	:	substrate concentration
SDS	:	Sodium Dodecyl Sulphate
SDS-PAGE	:	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis.
U/ml	:	Units per milli liter

1. INTRODUCTION

1. ENZYMES[1, 2, 3, 4]

Enzymes are proteins that catalyze (*i.e.* accelerate) chemical reactions. In enzymatic reactions, the molecules at the beginning of the process are called substrates, and the enzyme converts them into different molecules, the products. Almost all processes in a biological cell need enzymes in order to occur at significant rates. Since enzymes are extremely selective for their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathways occur in that cell.

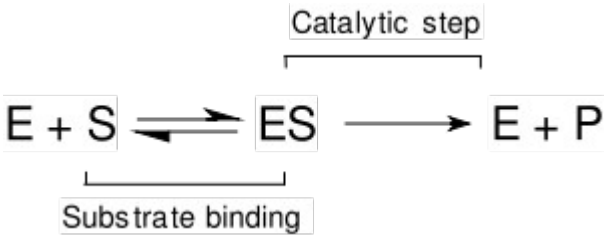
1.1. SPECIFICITY[3, 4]

Enzymes are usually very specific as to which reactions they catalyze and the substrates that are involved in these reactions. Complementary shape, charge and hydrophilic/hydrophobic characteristics of enzymes and substrates are responsible for this specificity. Enzymes can also show impressive levels of stereospecificity, regioselectivity and chemoselectivity.

Some of the enzymes showing the highest specificity and accuracy are involved in the copying and expression of the genome. These enzymes have "proof-reading" mechanisms. Here, an enzyme such as DNA polymerase catalyses a reaction in a first step and then checks that the product is correct in a second step. Similar proofreading mechanisms are also found in RNA polymerase, aminoacyl tRNA synthetases and ribosomes.

Some enzymes that produce secondary metabolites are described as promiscuous, as they can act on a relatively broad range of different substrates. It has been suggested that this broad substrate specificity is important for the evolution of new biosynthetic pathways.

1.2. KINETICS[3, 4]

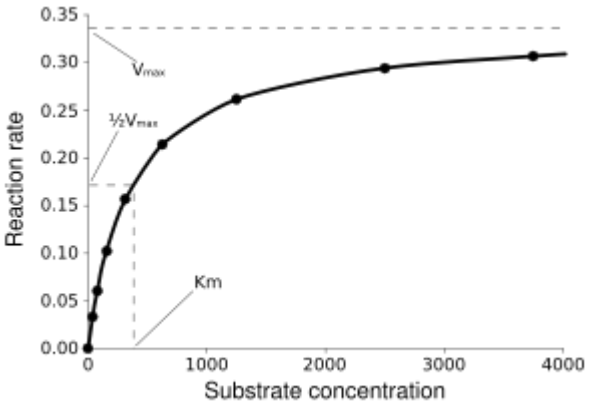


Mechanism for a single substrate enzyme catalyzed reaction. The enzyme (E) binds a substrate (S) and produces a product (P).

Enzyme kinetics is the investigation of how enzymes bind substrates and turn them into products. The rate data used in kinetic analyses are obtained from enzyme assays.

The major contribution of Henri was to think of enzyme reactions in two stages. In the first, the substrate binds reversibly to the enzyme, forming the enzyme-substrate complex. This is sometimes called the Michaelis complex. The enzyme then catalyzes the chemical step in the reaction and releases the product.

Figure 1: Saturation curve for an enzyme reaction showing the relation between the substrate concentration (S) and rate (v).



Enzyme rates depend on solution conditions and substrate concentration. Conditions that denature the protein abolish enzyme activity, such as high temperatures, extremes of pH or high salt concentrations, while raising substrate concentration tends to increase activity. To find the maximum speed of an enzymatic reaction, the substrate concentration is increased until a constant rate of product formation is seen. This is shown in the saturation curve on the right. Saturation happens because, as substrate concentration increases, more and more of the free enzyme is converted into the substrate-bound ES form. At the maximum velocity (V_{max}) of the enzyme, all the enzyme active sites are bound to substrate, and the amount of ES complex is the same as the total amount of enzyme. However, V_{max} is only one kinetic constant of enzymes. The amount of substrate needed to achieve a given rate of reaction is also important. This is given by the Michaelis-Menten constant (K_m), which is the substrate concentration required for an enzyme to reach one-half its maximum velocity. Each enzyme has a characteristic K_m for a given substrate, and this can show how tight the binding of the substrate is to the enzyme.

Another useful constant is k_{cat}, which is the number of substrate molecules handled by one active site per second.

The efficiency of an enzyme can be expressed in terms of k_{cat}/K_m. This is also called the specificity constant and incorporates the rate constants for all steps in the reaction. Because the specificity constant reflects both affinity and catalytic ability, it is useful for comparing different enzymes against each other, or the same enzyme with different substrates.

Michaelis-Menten kinetics relies on the law of mass action, which is derived from the assumptions of free diffusion and thermodynamically-driven random collision. However, many biochemical or cellular processes deviate significantly from these conditions, because of very high concentrations, phase-separation of the enzyme/substrate/product, or one or two-dimensional molecular movement. In these situations, a fractal Michaelis-Menten kinetics may be applied.

1.3. BIOLOGICAL FUNCTION[2, 4]

Enzymes serve a wide variety of functions inside living organisms. They are indispensable for signal transduction and cell regulation, often via kinases and phosphatases. They also generate movement, with myosin hydrolysing ATP to generate muscle contraction and also moving cargo around the cell as part of the cytoskeleton. Other ATPases in the cell membrane are ion pumps involved in active transport. Enzymes are also involved in more exotic functions, such as luciferase generating light in fireflies. Viruses can also contain enzymes for infecting cells, such as the HIV integrase and reverse transcriptase, or for viral release from cells, like the influenza virus neuraminidase.

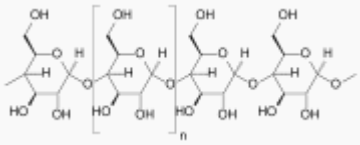
An important function of enzymes is in the digestive systems of animals. Enzymes such as amylases and proteases break down large molecules (starch or proteins, respectively) into smaller ones, so they can be absorbed by the intestines. Starch molecules, for example, are too large to be absorbed from the intestine, but enzymes hydrolyse the starch chains into smaller molecules such as maltose and eventually glucose, which can then be absorbed. Different enzymes digest different food substances. In ruminants which have a herbivorous diets, microorganisms in the gut produce another enzyme, cellulase to break down the cellulose cell walls of plant fiber.

Enzymes determine what steps occur in these pathways. Without enzymes, metabolism would neither progress through the same steps, nor be fast enough to serve the needs of the cell. Indeed, a metabolic pathway such as glycolysis could not exist independently of enzymes. Glucose, for example, can react directly with ATP to become phosphorylated at one or more of its carbons. In the absence of enzymes, this occurs so slowly as to be insignificant. However, if hexokinase is added, these slow reactions continue to take place except that phosphorylation at carbon 6 occurs so rapidly that if the mixture is tested a short time later, glucose-6-phosphate is found to be the only significant product. Consequently, the network of metabolic pathways within each cell depends on the set of functional enzymes that are present.

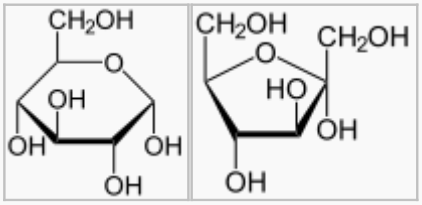
1.4. INDUSTRIAL APPLICATIONS[1, 4]

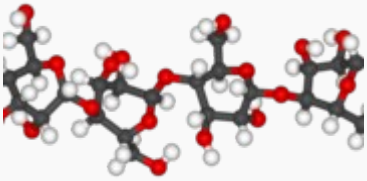
Enzymes are used in the chemical industry and other industrial applications when extremely specific catalysts are required. However, enzymes in general are limited in the number of reactions they have evolved to catalyse and also by their lack of stability in organic solvents and at high temperatures. Consequently, protein engineering is an active area of research and involves attempts to create new enzymes with novel properties, either through rational design or *in vitro* evolution.

Table 1: Industrial Applications of Beta-Galactosidase

Application	Enzymes used	Uses
Baking industry  alpha-amylase catalyzes the release of sugar monomers from starch	Fungal alpha-amylase enzymes are normally inactivated at about 50 degrees Celsius, but are destroyed during the baking process.	Catalyze breakdown of starch in the flour to sugar. Yeast action on sugar produces carbon dioxide. Used in production of white bread, buns, and rolls.
	Proteases	Biscuit manufacturers use them to lower the protein level of flour.
Baby foods	Trypsin	To predigest baby foods.

Brewing industry  Germinating barley used for malt.	Enzymes from barley are released during the mashing stage of beer production.	They degrade starch and proteins to produce simple sugar, amino acids and peptides that are used by yeast for fermentation.
	Industrially produced barley enzymes	Widely used in the brewing process to substitute for the natural enzymes found in barley.
	Amylase, glucanases, proteases	Split polysaccharides and proteins in the malt.
	Betaglucanases and arabinoxylanases	Improve the wort and beer filtration characteristics.
	Amyloglucosidase and pullulanases	Low-calorie beer and adjustment of fermentability.
	Proteases	Remove cloudiness produced during storage of beers.
	Acetolactatedecarboxylase (ALDC)	Avoid the formation of diacetyl
Fruit juices	Cellulases, pectinases	Clarify fruit juices
Dairy industry	Rennin, derived from the stomachs of young ruminant animals (like	Manufacture of cheese, used to

 Roquefort cheese	calves and lambs).	hydrolyze protein.
	Microbially produced enzyme	Now finding increasing use in the dairy industry.
	Lipases	Is implemented during the production of Roquefort cheese to enhance the ripening of the blue-mould cheese.
	Lactases	Break down lactose to glucose and galactose.
Meat tenderizers	Papain	To soften meat for cooking.
Starch industry  Glucose Fructose	Amylases, amyloglucosidases and glucoamylases	Converts starch into glucose and various syrups.
	Glucose isomerase	Converts glucose into fructose in production of high fructose syrups from starchy materials. These syrups have enhanced sweetening properties and lower calorific values than sucrose for the same level of sweetness.
Paper industry	Amylases, Xylanases, Cellulases and ligninases	Degrade starch to lower viscosity, aiding sizing and coating paper.

 A paper mill in South Carolina.		Xylanases reduce bleach required for decolorising; cellulases smooth fibers, enhance water drainage, and promote ink removal; lipases reduce pitch and lignin-degrading enzymes remove lignin to soften paper.
Biofuel industry  Cellulose in 3D	Cellulases	Used to break down cellulose into sugars that can be fermented.
	Ligninases	Use of lignin waste
Biological detergent Laundry soap	Primarily proteases, produced in an extracellular form from bacteria	Used for presoak conditions and direct liquid applications helping with removal of protein stains from clothes.
	Amylases	Detergents for machine dish washing to remove resistant starch residues.
	Lipases	Used to assist in the removal of fatty and oily stains.
	Cellulases	Used in biological

		fabric conditioners.
Contact lens cleaners	Proteases	To remove proteins on contact lens to prevent infections.
Rubber industry	Catalase	To generate oxygen from peroxide to convert latex into foam rubber.
Photographic industry	Protease (ficin)	Dissolve gelatin off scrap film, allowing recovery of its silver content.
Molecular biology	Restriction enzymes, DNA ligase and polymerases	Used to manipulate DNA in genetic engineering, important in pharmacology, agriculture and medicine. Essential for restriction digestion and the polymerase chain reaction. Molecular biology is also important in forensic science.

2. BETA-GALACTOSIDASE[5, 6, 7, 8, 9, 11]

The lactose-hydrolysing enzyme, β-galactosidase (β-D-galactoside galacto hydrolase, trivially lactase) has long been accepted as an important enzyme for dairy industry. β-galactosidase catalyze two reactions: it catalyses hydrolysis of lactose, the milk sugar into glucose and galactose and in some cases β-galactosidase is able to catalyze transglycosylation reactions. In dairy industry, β-galactosidase has been used to prevent crystallization of lactose, to improve sweetness, to increase the solubility of the milk product. Moreover, it has been used to produce low lactose containing food

products for low lactose tolerance people and for the utilization of whey, which would otherwise be an environmental pollutant. β-galactosidase was among the first hydrolyses to be discovered. β-D-Galactopyranosides, such as lactose, are thereby converted to galactooligosaccharides.

Enzyme preparations used to produce lactose free products are highly purified proteins. The more the protein purified, the higher its cost become. Therefore, the cost of the lactose-reduced milk is about 80% higher than the regular unhydrolysed milk.

The commercial enzymes used for lactose hydrolysis are β-galactosidase of diverse origins. Possible sources of the enzyme are: plants, animal organs, bacteria, yeasts (intracellular enzyme), fungi and moulds (extracellular enzyme). Among them bacterial sources are preferable because of ease of fermentation, high activities of the enzyme and good stability.

The most commonly found natural substrate for this enzyme is lactose, the main sugar of milk and several dairy products. Lactose, however, is not the only substrate. ONPG also acts as a good substrate. Some enzymes in this group hydrolyze alpha-L-arabinosides; some animal enzymes also hydrolyze beta-D-fucosides and beta-D-glucosides.

2.1. SOURCES OF β-GALACTOSIDASE[5, 6, 7, 8, 9, 11]

Many organisms have been selected due to their high levels of lactase activity for commercial use. The commercially exploited sources of β-galactosidase have been of microbial origin, mainly yeast and moulds. Even if yeasts (intracellular enzyme), fungi or molds (extracellular enzyme) are known to produce β-galactosidase, bacterial sources are preferable because of ease of fermentation, high activities of enzyme and good stability.

Due to secretion abilities, stability and simplicity of purification of the enzyme, still the Bacillus strain is one of the common strains that has been used for lactase production. The most common bacilli used in the industry are *B. licheniformis*, *B. amyloliquefaciens* and *B. subtilis*. Regardless of the source organism, long-term stability of the enzyme continues to be a problem. There is a definite need for β-galactosidase that is stable at high and low temperatures and could be approved as GRAS for hydrolysis of lactose in milk and other dairy products.

Table 2: The list of organisms that produce lactase

Category	Source	Product Name	Comments
Yeast	*Candida pseudotropicalis*	Neutral	Used for hydrolysis of whey
	Kluyveromyces	Lactase	Used for hydrolysis of whey
	(Sacchoramoyces) fragilis	Hydrolact	Used for hydrolysis of whey
	Kluyveromyces lactis	Maxilact	
Animal organs	Intestine Brain and skin		
Bacteria	*megaterium* *Escherichia coli*		
	Lactobacillus acidophilus	Acidophilas	It contains lactase enzyme
	Lactobacillus	(Wakunaga	
	Lactobacillus belatericus *Lactobacillus crispatus* *Lactobacillus helveticus* *Lactobacillus pentosus* *Streptoccus lactis* *Streptococcus thermophilus* *Thermus aquaticus*	Probiotics)	
Fungi	*Aspergillus flavus* *Aspergillus foetidus*		
	Aspergillus niger	Valio	Used for hydrolysis of whey
	Aspergillus oryzae	Lactase F	
	Aspergillus phoenicis	Amano	
	Curvularia inaqualis *Mucor meibei* *Mucor pusillus* *Neurospora crassa*	Enzeco	

Plants	Almonds Apricot Coffee berries Kefir grains Peach		

Table 3: Properties of Lactase

Sources	pH optimum	Temperature Optimum	Molecular weight (kDa)	Activating ions. Other remarks
A.niger	3,0 -4,0	55-60	124	
A. oryzae	5,0	50-55	90	Mn^{+2}, K^{+}
K. fragilis	6,9-7,3	37	201	Mn^{+2} , Na^{+}
K. lactis	7,2	35	135	K^{+} , Na^{+}
E.coli	6,2-7,1	40	540	
L. thermophilus	3,4-4,3	55-57	530	
C. inaegualis	6,0	30-55		High activity for
B.circul ans	6,8	60-65		skim milk
Bacillus sp.	6,5-7,5	65		
L .bulgaricus		42-45		
S. thermophilus		45-55	500-600	

3. LACTIC ACID BACTERIA[11, 13]

Residues of cheese in an Egyptian pot dating from 2300 BC and passages in the Bible (Anonymous) indicate that lactic acid bacteria have been used for the fermentation and preservation of human food stuffs for at least 4-5 millennia. Members of the lactic acid bacteria have been defined on the basis of cell morphology, DNA base composition, and type of fermentative metabolism. Recently application of molecular genetic techniques to determine the relatedness of food-associated lactic acid bacteria has resulted in significant changes in their taxonomic classification. The lactic acid bacteria associated with foods, now include species of the genera *Carnobacterium*, *Oenococcus, Tetragenococcus, Vagococcus,*

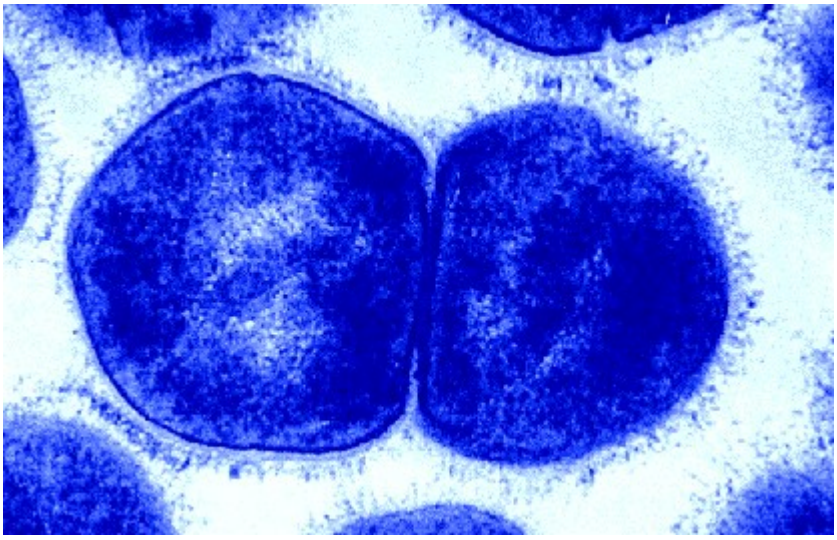
Lactococcus, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Leuconostoc*, and *Pediococcus*.

The lactic acid bacteria are gram-positive, usually non-motile, nonsporulating, catalase negative, cocci, coccobacilli, or rods bacteria that produce lactic acid as a major or sole product of fermentative metabolism. Lactic acid bacteria have less than 55 mol% G+C content in their DNA. Therefore, they are called low G-C gram positive bacteria. Members of this group lack porphyrins and cytochromes, do not carry out electron transport phosphorylation, and hence obtain energy only by substrate level phosphorylation. Lactic acid bacteria are aerotolerant anaerobes. Most lactic acid bacteria obtain energy only from the metabolism of sugars and related fermentable compounds and hence are usually restricted to habitats in which sugars are present.

One important difference between subgroups of the lactic acid bacteria lies in the nature of the products formed during the fermentation of sugars. One group, called homofermentative, produces virtually a single fermentation product, lactic acid, whereas the other group, called heterofermentative, produces mainly ethanol and CO_2 as well as lactate.

3.1. STREPTOCOCCUS[14, 15]:

Figure 2: Streptococcus: chains of nearly spherical bacteria



The genus *Streptococcus* is comprised of a wide variety of both pathogenic and commensal gram-positive bacteria which are found to inhabit a wide range of hosts, including humans, horses, pigs and cows. Within the host, streptococci are

often found to colonize the mucosal surfaces of the mouth, nares and pharynx. However, in certain circumstances, they may also inhabit the skin, heart or muscle tissue.

Pathogenic streptococci of man include *S. pyogenes, S. pneumoniae*, and *S. faecalis*. Among the pathogenic hemolytic streptococci, *S. pyogenes*, or group A streptococci, has been implicated as the etiologic agent of acute pharyngitis ("strep throat"), impetigo, rheumatic fever, scarlet fever, glomerulonephritis, and invasive fasciitis. A peptidoglycan cell wall, containing muramic acid and glucosamine, is common to all streptococci. Pathogenic streptococci have also been classified into groups according to carbohydrate antigens associated with the cell wall. Group A streptococci may be further subdivided according to the variety of M protein expressed on the cell surface.

3.2. STREPTOCOCCUS THERMOPHILUS STRAIN[13, 15, 19]:

Streptococcus thermophilus is of major importance for the food industry since it is massively used for the manufacture of dairy products and it is considered as the second most important industrial dairy starter after *Lactococcus* (*Lc.*) *lactis*. This bacterium belongs to the group of the thermophilic lactic acid bacteria and is traditionally used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* or *Lb. helveticus* for the manufacture of yoghurt.

S. thermophilus is closely related to *Lc. lactis*, but it is even more closely related to other streptococcal species comprising several deadly human pathogens (e.g. *S. pneumoniae, S. pyogenes, S. agalactiae*), which cause for example pneumonia, bacterial sepsis or meningitis. *S. thermophilus* is also related to *S. mutans*, the most important pathogen in tooth decay. Nevertheless, *S. thermophilus* is a “generally recognized as safe” (GRAS) species and over 10^{21} live cells are ingested annually by the human population.

The comparison of *S. thermophilus* genomes with published genomes of streptococcal pathogens highlights its relatedness to pathogenic species but also reveals that the most important determinants for pathogenicity are either absent or present as pseudogenes, unless they encode basic cellular functions. This reinforced our view that the massive consumption of this bacterium by humans entails no health risk. Comparative genomics also revealed that evolution has shaped the *S. thermophilus* genome mainly through loss-of-function events, even if lateral gene

transfer played an important role, revealing that the dairy streptococcus has followed an evolutionary path divergent to that of pathogenic species due to its adaptation to the rather narrow and well-defined ecological niche, milk.

The wealth of genomic information will not only aid our understanding of the molecular biology and physiology of *S. thermophilus*, but will also facilitate selection of appropriate *S. thermophilus* starters by the food industry, as well as enhance our insight in pathogenic processes involving streptococci.

Table 4: Characteristics of *Streptococcus*

S.No	Characteristics	Streptococcus
1	Predominant arrangement	Chains, pairs
2	Capsule/slime layer	Positive
3	Habitat	Mouth, respiratory tract
4	Growth at 45°C	Variable
5	Growth at 10°C	Variable
6	Growth at 6.5% NaCl broth	Variable
7	Growth at pH 9.6	Variable
8	Hemolysis	usually beta (pyogenic) or alpha (oral)
9	Serological group	variable (A-O)
10	Mol % (G+C) (normal range)	34-46

In contrast to the other lactic acid bacteria present in starter cultures, *Streptococcus thermophilus* possesses a urease, which converts urea into ammonia and carbon dioxide. Even if this enzyme is not required for the growth of *S. thermophilus* in milk, we observed recently that its inhibition by fluorofamide decreased the growth of most of the strains tested. One hypothesis to explain this result is that ammonia and carbon dioxide produced from urea could be used in several biosynthesis routes, such as amino acid production. In the present study, we investigated the importance of glutamine synthesis for the growth of *S. thermophilus* in milk and its relation with the catabolism of urea.

Streptococcus thermophilus grows more rapidly at first and renders the milk anaerobic and weakly acidic. A variety of gram positive bacteria produce lactic acid as their major or sole fermentation product and are collectively called as Lactic Acid

Bacteria. The genera are distinguished primarily based on phenotypic properties such as oxygen relationships, cell arrangement, the presence of catalase and cytochromes, and peptidoglycan structure.

Streptococcus thermophilus and *Lactobacillus bulgaricus* are alive in yoghurt. Genome analysis has also shown that adaptation to milk has caused the acquisition of genes which have become useful to *S. thermophilus* in its new ecological niche. Thus the bacterium can draw energy necessary for its growth from the sugar in the milk (lactose).

The genome of *S. thermophilus* contains the genes necessary for the synthesis of methionine. It is used extensively in the production of yoghurt, cheese, fermented dairy products, and strain improvement through genetic manipulation.

To apply recombinant DNA technology to *S. thermophilus*, a suitable cloning vector is required. The plasmid discovered in different strains of *S. thermophilus* have large spectra, some carry genes responsible for a variety of functions such as sugar metabolism, proteolysis, citrate fermentation, and bacteriocin production as well as resistance to bacteriophage, inorganic ions, and antibiotics.

Moreover, natural strains that are resistant to antibiotics and have stable plasmid DNA might be used in industry for high quality production. Antibiotic resistant strains may be selected for probiotic use in the human gastrointestinal system as a barrier to infection.

4. HYDROLYSIS MECHANISM[11, 49]

The mechanism of lactose hydrolysis was described by using lactase obtained from *E.coli*. The reaction mechanism proposed in these articles was that the active site of β-galactosidase contains the cysteine and histidine amino acids which function as proton donor and proton acceptor, respectively. Cysteine contains the sulphydryl group (acting as a general base) acted as proton donor and histidine residues (contains imidazole group as a substructure) acted as nucleophile site to facilitate splitting of the glycosidic bond, respectively, during the enzymatic hydrolysis procedure. Proposed mechanism of lactose hydrolysis by β-galactosidase is represented. The galactosyl transfer reaction is shown.

Recently a new active site for β-galactosidase has been suggested and widely accepted. Glutamic acid residue was suggested as the new active site. The β-galactosidase from a variety of microbial origins has two glutamic acid residues (such

as Glu[482] and Glu[551]) as the proton donor and the nucleophile/base at the same time in the enzymatic reaction. The first step is the enzyme-galactosyl complex formation and simultaneous glucose liberation. In the second step, the enzyme-galactosyl complex is transferred to an acceptor containing a hydroxyl group. While in a diluted lactose solution, water (rather than other sugars such as glucose, lactose) can be more competitive to be an acceptor, therefore galactose is formed and released from the active site. On the other hand, in high lactose content solution, lactose molecule has more changes to act as the acceptor, binding with the enzyme-galactose complex to form oligosaccharides.

Figure 3: Proposed mechanism of lactose hydrolysis by β-galactosidase. Sulphydryl group acted as proton acceptor and imidazole group acted as proton donor.

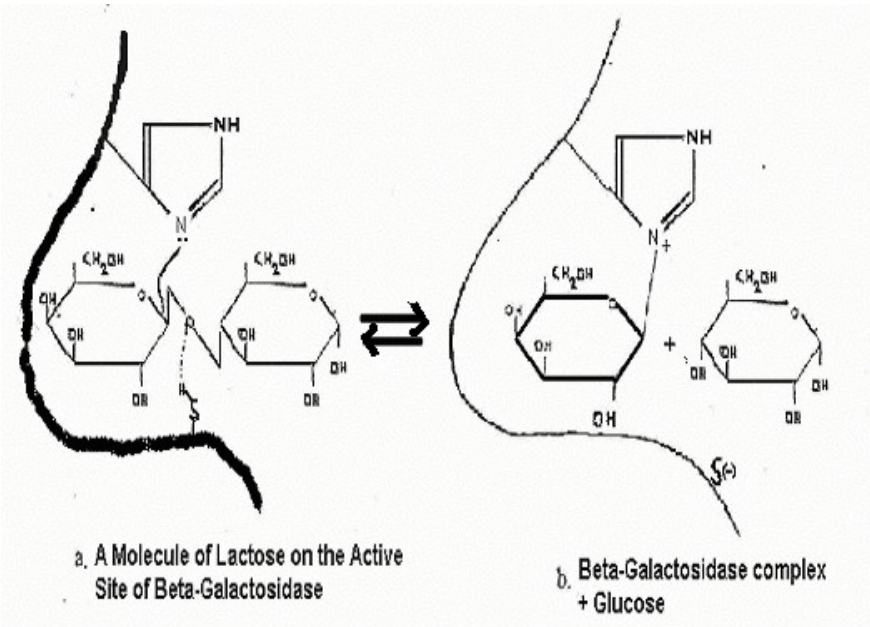


Figure 4: Proposed mechanism of galactosyl transfer reaction catalyzed by β-galactosidase.

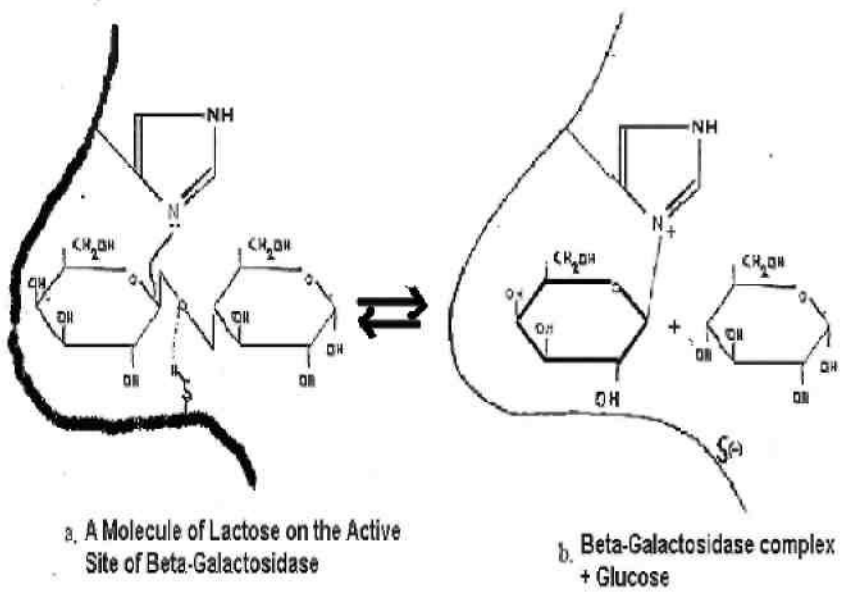
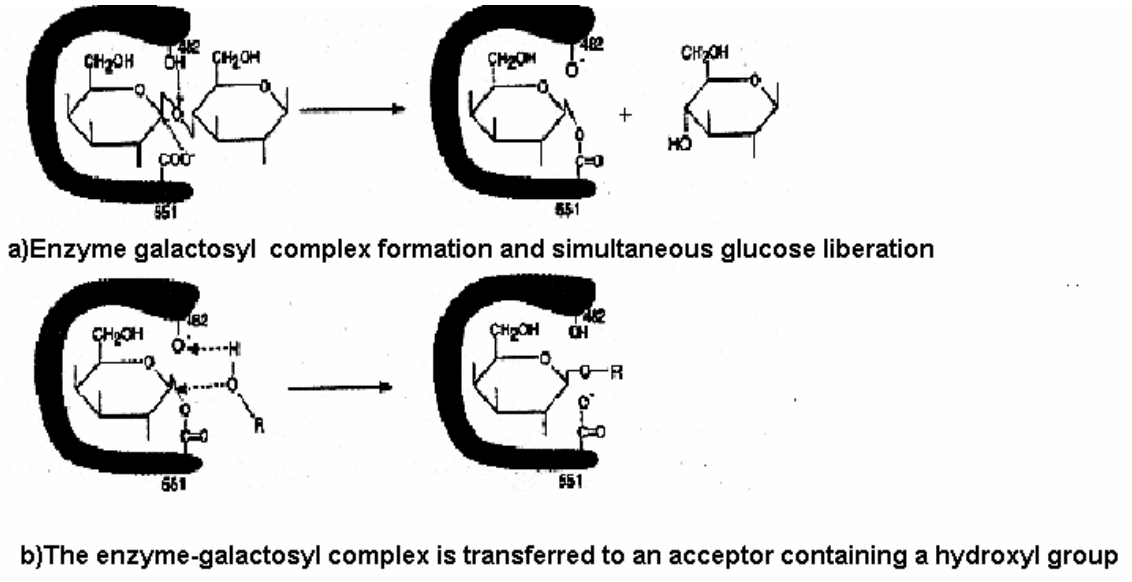


Figure 5: Schematic mechanism of the lactose hydrolysis by β-galactosidase. This mechanism has been suggested recently. Glutamic acid functions at active sites as the proton donor (Glu^{482}) and the nucleophile/base (Glu^{551}) in the enzymatic reactions.



Although the enzymes derived from various microbial origins have different properties, such as molecular weight, protein chain length, and the position of the active site; it has been found recently that β-galactosidase from different sources have the same amino acid residue, glutamic acid, as their catalytic site.

4.1. MECHANISM OF LACTOSE TRANSPORT IN LACTIC ACID BACTERIA

Most bacterial cells have the capacity to utilize several carbohydrates as carbon and energy source and possess various transport proteins and catabolic enzymes for the metabolism of the different carbohydrates. The systems by which the carbohydrate molecules are transported can be subdivided into 3 classes that differ in their mechanism of energy coupling: (a) primary transport systems, (b) secondary transport systems and (c) phosphotransferase systems (PTS). These different mechanisms of transport have been observed for a wide variety of sugars but only those that mediate lactose transport in lactic acid bacteria will be described in detail[11, 17].

In case of lactose transport, generally secondary transport systems and PEP–PTS are used in lactic acid bacteria. These two mechanisms use different enzymes to catalyze the hydrolyzation reaction of lactose. β-galactosidase enzyme is the marker enzyme for secondary transport system, on the other hand P-β-galactosidase is marker enzyme of phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS). In the secondary transport system, lactose is not chemically modified. But in PEP-PTS case, translocation of lactose across the cytoplasmic membrane is coupled to the chemical modification of the molecule, ie. transport followed with phosphorylation of the lactose by PTS.

4.1.1. PRIMARY TRANSPORT SYSTEM

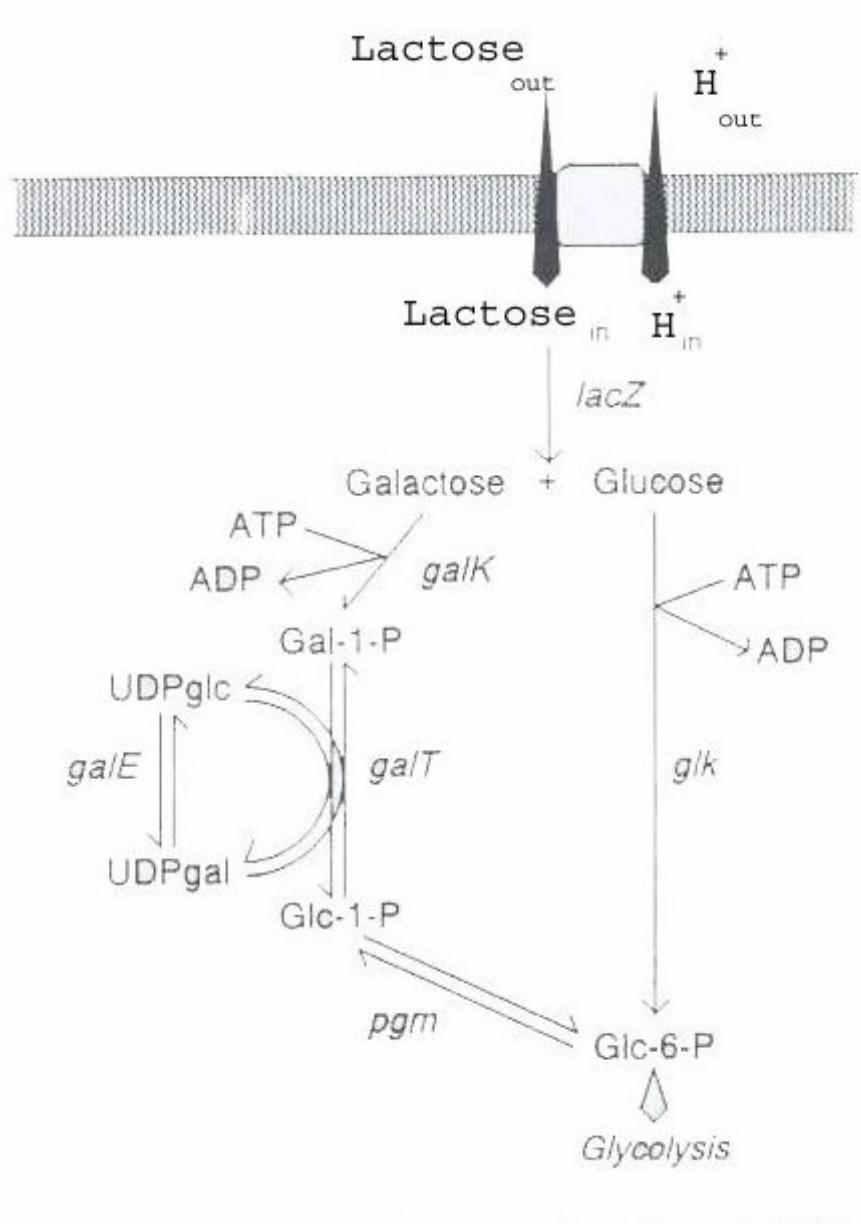
Primary transport systems use the energy driven by the hydrolysis of an energy-rich chemical bond for translocation of a sugar. In the lactic acid bacteria, the ATP-binding cassette transporters are the most abundant class of transporters in primary transport systems. ATP-binding cassette transporters are used not only to accumulate substrates and compatible solutes, but also to excrete unwanted products such as drugs. The number of ATP molecules hydrolyzed per solute taken up by the transport ATPases is most likely 1-2 which makes these transporters energetically expensive as compared to the ion-linked transporters, exchange systems and PTS. Downstream of generally accepted (putative) lactose transport ATPase genes of *Lc lactis*, two translationally coupled genes (*lacL* and *lacM*) have been found to encode a funtional β-galactosidase (LacZ) of *S thermophilus*, *Lb bulgaricus* and *E.coli.*

4.1.2. SECONDARY TRANSPORT SYSTEM

In the secondary transport system the translocation of a sugar molecule is supplied by the sugar concentration gradient, and, if another molecule is co- or counter transported with the carbohydrate, the (electro-) chemical gradient of this coupling molecule, *i.e.* accumulation is achieved by the downhill movement of another molecule.

In *Lactobacillus delbrueckii* subsp. *bulgaricus* as well as in *Streptococcus thermophilus*, lactose is known to be transported by the secondary transport system. These lactose transporters turn out to be specific not only for lactose (β-galactoside) but also for melibiose (α-galactoside), galactose (monosaccharide) and to a lesser extent raffinose (trisaccharide). The genes encoding the lactose (galactoside) transport proteins (LacS) of *S. thermophilus* and *L. bulgaricus* have been cloned, characterized and functionally expressed in E coli. The LacS permease works in antiport with the galactose (non-metabolizable in most *Streptococcus* strains) internally released from lactose by β-galactosidase, and also (at a lower efficiency) in symport with H^+. It was recently suggested that the galactoside transporter of *S. thermophilus* is a strict lactose/galactose antiporter is not correct. Although the lactose/galactose exchange reaction may be favoured under many conditions, the exchange mode simply reflects partial steps, forward and backward reactions with no net proton translocation, of a complete translocation cycle involving sugar and proton uptake on one side and release on the other side of the membrane, and reorientation of loaded and unloaded substrate binding sites. The lactose transport genes (*lac*S) of *S. thermophilus* and *Lb bulgaricus* are organized in an operon that also contains the β-galactosidase gene (*lac*Z).

Figure 6. Galactoside-H^+ symport and Leloir pathway. lacZ, β-galactosidase; galK, galactokinase; galT, UDPglucose: galactose 1-phosphate uridylyl transferase; galE, UDPglucose 4-epimerase; pgm, phosphoglucomutase; glk, glucokinase.



4.1.3. PHOSPHOENOLPYRUVATE DEPENDENT PHOSPHOTRANSFERASE SYSTEM (PEP-PTS)

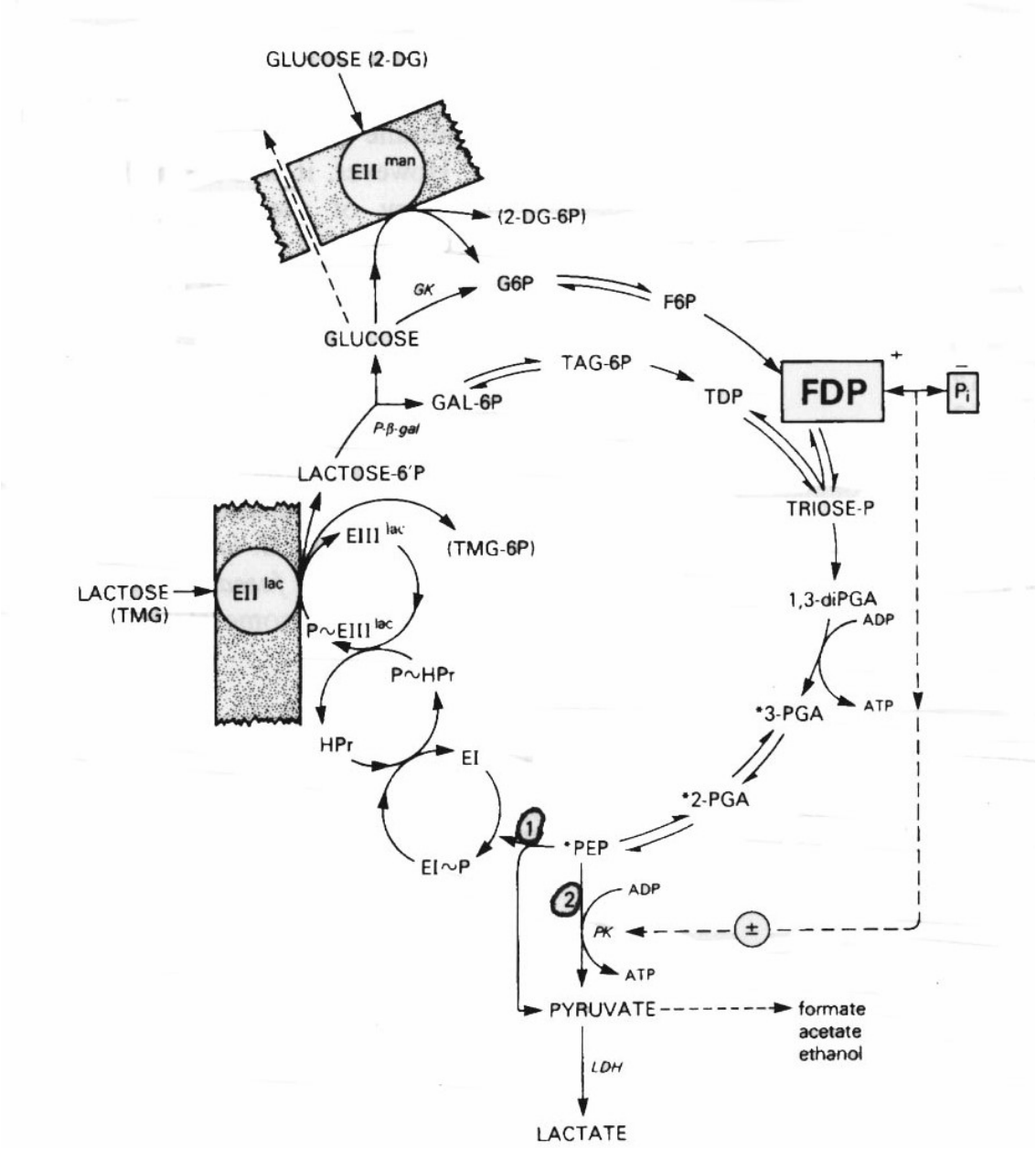
PEP-PTS system is involved in both the transport and phosphorylation of a large number of carbohydrates. Regardless of the organism or carbohydrate, all PTSs that have been characterized catalyze the following overall process:

Phospho(P)-enolpyruvate (in) + carbohydrate (out) ---------- ^{PTS}---> pyruvate (in) + carbohydrate P(in)

Carbohydrate phosphorylation is coupled to its translocation across the membrane, the energy for these processes being provided by the glycolytic intermediate PEP. In most gram-positive bacteria and in a few plasmid containing strains of enteric bacteria, galactose and the disaccharide lactose are PTS carbohydrates. Lactose specific PTS and P-β-galactosidase (P-β-gal) has only been described in Gram-positive bacteria belonging to the genera *Staphylococcus*, *Streptoccocus*, *Lactococcus* and *Lactobacillus.* Certain fundamental differences are found between genera regarding their gene order, regulatory elements, accompanying genes and genetic location.

This multicomponent phosphotransfer system consists of the two general cytoplasmic proteins (enzyme I (EI) and HPr (heat sensitive protein)) and two sugar-specific proteins. One of the sugar-specific pair (III^{sugar}) may be cytoplasmic or loosely associated with the cell membrane, while the other is an integral membrane protein (II^{sugar}) which recognizes, binds and mediated translocation of substrate.

Figure 7: Diagrammatic representation of the lactose-PTS and glycolysis cycle in S. lactis. Number 1 and 2, show distribution of PEP to the lac-PTS or to phosphokinase (PK), respectively. The symbols (+) and (-), indicate positive or negative effectors of PK; constituent of the PEP-potential in starved cells.



5. Industrial Applications of β-Galactosidase[5, 6, 7, 8, 9, 11]

a). β-galactosidase has catalytic property to hydrolyze lactose into glucose and galactose. Due to its hydrolyzing property of lactose, it has been used for new milk and fermented milk products. Potential beneficial effects on the assimilation of foods containing lactose, as well as the possible technological and environmental advantages of industrial application are listed.

b). Lactose is used for improving sweetness, the solubility of the milk product, broader fermentation possibilities, more ready fermentation of these sugars, and

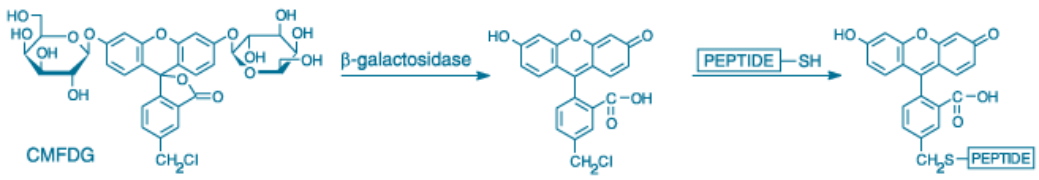
reduced lactose concentration with associated diminished possibility of lactose crystallization.

c). Low lactose milk, dairy products and yoghurt are consumed by lactose malabsorbers whose problem is generally related with lactase insufficiency. Low lactose milk, dairy products and yoghurt consumption generally decrease the intolerance symptoms arising due to lactose consumption.

d). Lactase has been used in cheese industry for a long time. Whey is the relatively clear supernatant that remains after the coagulated casein is separated from the milk for cheese making. Its lactose content is 4.2 to 4.4 %. Modification of whey by lactose hydrolysis could lead to new pathways toward practical and economic use for whey that is presently a waste product.

e). The sweet syrup prepared from whey by lactose hydrolysis can be used as a source of sugar and, in some cases, of protein in bakery products, in confectionery, in soft drinks, in ice cream, in feedstuffs for cattle instead of molasses, in dairy desserts, or as basis for further fermentation to alcohol. Hydrolyzed demineralized lactose syrup was produced by Valio.

f). Sequential ß-galactosidase hydrolysis and peptide conjugate formation of CMFDG, a component of the DetectaGene Green CMFDG *lacZ* Gene Expression Kit.



Some important applications of β-Galactosidase are as follows;

i). Elimination of lactose intolerance.

ii). Formation of galacto-oligosaccharides during lactose hydrolysis for favour the growth of intestinal bacterial microflora.

iii). Improvement in the technological and sensorial characteristics of dairy foods.

iv). Greater biodegradability of whey.

v). Recombinant purified Beta-Galactosidase (E. Coli) protein used for ELISA test.

vi). Beta-Galactosidase is used as a reporter gene in recombinant DNA technology and immunology.

6. Gram of ammonium sulphate added to 1L solution[20]

a). At M1 molar to take it to M2 molar

$$g = \frac{533\ (M2-M1)}{4.05-\ 0.3\ M2}$$

b). At S1 % saturation to make it to S2 % saturation.

$$g = \frac{533\ (S2-S1)}{100-\ 0.3S2}$$

Specific activity and % yield of enzyme:

The purity of enzyme preparation is expressed by its specific activity which relates its total catalytic activity to the total amount of protein present in the preparation.

$$\text{Specific activity} = \frac{\text{Total units of enzyme}}{\text{Total amount of protein}}$$

A successful fractionation is recognized by a fraction with a high specific activity and hence fold purification and high yield were,

$$\text{Yield} = \frac{\text{Units of enzyme in fraction}}{\text{Units of enzymes in original preparation}}$$

2. LITERATURE REVIEW

- **Klaus Weber *et al.,*. (1969)[41];** studied forty proteins with polypeptide chains of well-characterized molecular weights by polyacrylamide gel electrophoresis, following the procedure of Shapiro, Vifiuela, and Maizel. A smooth curve was obtained when the electrophoretic mobilities were plotted against the logarithm of the known polypeptide chain molecular weights. The results showed that the method can be used to determine the molecular weights for a wide variety of proteins.

- **Barry G. Hall *et al.,* (1977)[52];** found *Klebsiella* strain RE 1544 contained two lac operons, one on the chromosome and other on a lac-plasmid. A mutant of RE 1544 was found to synthesize a Beta-Galactosidase that hydrolyzed ONPG but not lactose. Synthesis of this Bgase-III was induced by lactose but not by isopropyl-1-thio-ß-D-galactopyranoside or methyl-ß-D-thiogalactopyranoside. In both the regulation of synthesis and substrate specificity, Bgase-III strongly resembled the ebg° enzyme of E. coli.

- **Sonia A. De Bales *et al.,* (1979)[8];** produced Beta-Galactosidase from *Candida pseudotropicalis* grown in deproteinized whey. Maximum enzyme production in 2% whey was obtained by supplementation with 0.15% yeast extract, 0.1% ammonium sulphate, and 0.05% potassium dihydrogen phosphate. Optimum pH and temperature for enzyme activity were 6.2 and 47 °C. The enzyme had a K_m for ONPG of 3.06 x 10^{-3} M and V_{max} was estimated as 6.63 x 10^{-3} M per minute. It hydrolyzed 50 and 100% of lactose in whey and milk within 4 and 5 h, respectively at 37 °C.

- **Macris B. J. *et al.,*. (1981)[6];** prepared extracellular lactase as an ethanol precipitate from *Fusarium moniliforme* grown on whey. The enzyme functioned optimally at pH 3.8-5 and at 50 to 60 °C on both ONPG and lactose respectively. At optimum conditions, 50% of the lactose in whey was hydrolyzed by 10 U of this enzyme in 50h.

- **Basil J. Macris *et al.,* (1982)[7];** found *Alternaria alternata* excreted elevated quantities of thermostable Beta-Galactosidase enzyme when grown in whey medium. Optimum cultural conditions for enzyme production were a whey, lactose concentration of 6% 0.050 M ammonium sulphate, an inoculum size of 10^3 conidia per ml, and a cultivation time at 28 to 30 °C of 5 days. The maximum yield observed was 280 nanokatals of hydrolyzed ONPG per gm of whey lactose, was comparable to maximum yields reported for certain commercial fungi. Optimum pH and temperature were 4.5 and 60-70 °C respectively. These properties made the enzyme suitable for processing acid and less acid dairy products and by-products.

- **Maria De Fatima Somerlate Barbosa *et al.,* (1985)[9];** tested two strains of *Kluyveromyces fragilis* and one of *Kluyveromyces lactis* for their abilities to produce Beta-Galactosidase in cheese whey. *Kluyveromyces fragilis* 145 was selected for its higher production. Addition of ammonium sulphate (0.3%) and yeast extract (0.1%) increased cell mass and enzyme yield. The harvested yeast cells were ethanol-and-acetone- permeabilized to enhance lactose hydrolysis by Beta-Galactosidase.

- **Jennifer Loveland *et al.,* (1994)[33];** isolated and characterized three psychrotrophic strains with Beta-Galactosidase activities. The isolates were gram-positive, catalase-positive, obligate aerobes. The cell cycle morphology and cell wall composition suggest that the isolates are members of the genus *Arthrobacter*. Electrophoresis of extracts from the isolates in nondenaturing polyacrylamide gels detected at least two protein bands that hydrolyzed X-Gal, suggesting the presence of Beta-Galactosidase isozymes.

- **Dave R. I. *et al.,* (1996)[22];** evaluated 15 medias to determine their suitability for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, and *Bifidobacteria* using 5 to 6 strains of each of the four groups of organisms. *Streptococcus thermophilus* agar was found to be suitable for selective enumeration of *Streptococcus thermophilus* under aerobic incubation at 37 ºC for 24 h. Other media that were evaluated in this study were not suitable for selective enumeration.

- **Ramana Rao M. V. *et al.,* (1997)[5];** extracted Beta-Galactosidase from *Streptococcus thermophilus* grown in deproteinized whey. Cultural conditions optimum for enzyme production were pH 7, 40 °C, and 24h. Proteose peptone, corn steep liquor, inorganic nitrogen, and phosphorous sources produced insignificant increase in enzyme production. Enzymatic hydrolysis for reduction of lactose content in aqueous solution and in skimmed milk was studied.

- **Shuichi Yanahira *et al.,* (1998)[45];** investigated structures of acidic oligosaccharides synthesized by a transglycosylation reaction by *Bacillus circulans* Beta-Galactosidase, using lactose as the galactosyl donor and N-acetylneuraminic acid and glucuronic acid as the acceptors. Acidic oligosaccharides were purified by anion exchange chromatography and charcoal chromatography. MS and NMR studies performed indicated that the acidic oligosaccharides from NeuAc were Galß-(1-8)-NeuAc, Galß-(1-9)-NeuAc, and those from GlcUA were Galß-(1-3)-GlcUA and Galß-(1-4)-GlcUA.

- **Taro Kiso *et al.,* (2000)[48];** synthesized p-hydroxybenzoyl beta-galactose chemically to examine the hydrolytic activity of beta-galactosyl ester linkage by Beta-Galactosidases. The enzymes from *E. coli* and *A. oryzae* hydrolyzed pHB-Gal with almost the same rates as pNB-Gal, pH-activity profiles, inhibition analysis, and kinetic properties of the enzymic reaction on pHB-Gal suggested that Beta-Galactosidase had only one active site for hydrolysis of both galactosyl ester and galactoside.

- **Hoyoux A. *et al.,* (2001)[32];** isolated purified Beta-Galactosidase from Antarctic gram-negative bacterium *Pseudoalteromonas haloplanktis* TAE 79. The nucleotide and the NH_2 - terminal amino acid sequence of the enzyme indicated that Beta-Galactosidase subunit was composed of 1,038 amino acids with a calculated M_r of 118,068. *P. haloplanktis* Beta-Galactosidase was expressed in *E. coli* and the recombinant enzyme displayed properties identical to those of the wild-type enzyme. Assays of lactose hydrolysis in milk suggested that cold-adapted Beta-Galactosidase could be used to hydrolyze lactose in dairy products processed in refrigerated plants.

- **Ming-Ni Hung *et al.,* (2001)[42];** two genes encoding Beta-Galactosidase isoenzymes, ß-gal II and ßgal III, from *Bifidobacterium infantis* HL 96 were revealed on 3.6 and 2.4 – kb DNA fragments, respectively by nucleotide sequence analysis. The amino acid sequences of ß-gal II and ßgal III were homologous to those found in the lacZ and the lacG families, respectively. The molecular masses were estimated by SDS-PAGE. The structure of the major trisaccharide produced by ßgal III catalysis was characterized as 3-galactosyl lactose.

- **Yoshiyuki Ito *et al.,* (2002)[51];** cloned lacA coding for Beta-Galactosidase from the genomic DNA of *Aspergillus oryzae* RIB40. There were 9 exons in lacA and the coding region of 3,015 bp encoded a protein of 1,005 aa with a deduced molecular mass of 109,898. Approximately 10 copies of lacA under control of *A. oryzae* glaA promoter were integrated into the chromosome of *A. oryzae* M-2-3. The recombinant strain expressed more than 700-fold of the Beta-Galactosidase activity as compared to the wild type strain under induction by maltose.

- **Graciette Matioli *et al.,* (2003)[40];** determined the kinetic modeling of the lactose hydrolysis and the operational stability of the enzyme Beta-Galactosidase of *Kluyveromyces fragilis*. For the operational stability both lactose concentrations (5 and 10%) were studied in the presence and absence of buffer. The experimental results showed that the buffer led to enzyme inactivation. In the kinetic modeling, the hydrolysis reaction was led to 40 °C/7h. The adjusted model concluded that it was necessary to use the quantity of 3450 LAU/L of the enzyme to obtain the hydrolysis from 70-80% of milk lactose in 2-3 hours of reaction.

- **James A. Coker *et al.,* (2003)[37];** isolated a psychrophilic gram-positive organism from Antarctic dry valley soil. It utilized lactose, had a rod-coccus cycle, and contained lysine as the diamino acid in its cell wall. The 16S ribosomal DNA sequence showed that it was phylogenetically related to other *Arthrobacter* species. A gene (bgaS) encoding a family 2 Beta-Galactosidase was cloned from this organism into an *E. coli* host. The enzyme was cold-active and heat-labile. Kinetic studies using ONPG showed that bgaS with

and without a His tag had greater catalytic activity at and below 20 °C than LacZ Beta-Galactosidase. Comparisons of family 2- Beta-Galactosidase amino acid and modeling studies with the LacZ structure did not mimic suggested trends for conferring enzyme flexibility at low temperatures.

- **Satoshi Kaneko *et al.,* (2003)[31];** purified Beta-Galactosidase by lactosyl-Sepharose 4B and Sephacryl S-200 column chromatographies from the cultured medium of a rice-cell suspension. The purified enzyme appeared as 47 KD and 40 KD polypeptides on SDS-PAGE and had a specific activity of 65.1 units/mg. The enzyme released galactose from galactoxyloglucan and pectic galactans.

- **Akihiro Nakamura *et al.,* (2003)[49];** found the fruit extracts of ripening Japanese Persimmon contained a number of glycosidases and glycanases. Among them, Beta-Galactosidase appeared to be most significant and the activity increased in parallel with tissue ripening. These results suggest that the ripening of Persimmon was caused by the solubilisation of pectic polysaccharide by endo-type glycanases and exo-type glycosidases. Beta-Galactosidase seemed to play a major role in ripening the fruit.

- **Belma Aslim *et al.,* (2004)[19];** isolated 34 *Streptococcus thermophilus* strains from yoghurt samples from different regions of Turkey. These strains were examined for antibiotic resistance patterns and plasmid carriage. Also 7 strains did not contain any plasmid DNA, while other strains included plasmid DNA ranging 1-5. An interaction was observed between the resistance to antibiotics and the occurrence of plasmids in some strains. The resistance to most antibiotics was determined in 3 strains containing 5 plasmid DNAs. However, no correlation was observed between the resistance to antibiotics and the occurrence of plasmids in some strains.

- **Simova E. D. *et al.,* (2004)[44];** studied about the production of exopolysaccharides by lactose-negative yeast and a yoghurt starter co-cultivated in a neutral substrate containing lactose. The mixed culture was cultivated in cheese whey ultrafiltrate in a MBR AG fermentor. The yoghurt starter synthesized neutral exopolysaccharides, while the mixed culture, yeast

+ yoghurt starter produced acidic exopolysaccharides containing uronic acid (6%). The present findings propose an alternative use of whey ultrafiltrate as a cost-effective carbohydrate substrate and suggest that lactose-negative yeast *Rhodotorula rubra* have industrial application as producers of exopolysaccharides.

- **Wan-Taek Im *et al.,* (2004)[35];** carried out a taxonomic study on a bacterial strain designated as JIP2^T isolated from a soil sample mixed with rotten rice straw. It was a gram-negative, aerobic, motile, rod shaped bacterium, and it grew well on nutrient agar medium. The G+C content was 65.3 mol%. The major ubiquinone was Q-8 and there were branched fatty acids. The 16 S rDNA sequence of strain JIP2^T showed 96.4% sequence similarity to that of *Rhodanobacter lindaniclasticus* RP 5575^T. They proposed the name *Rhodanobacter fulvus* sp. nov. for strain JIP2^T, which is a Beta-Galactosidase producing strain.

- **Hidetaka Nagatomo *et al.,* (2005)[36];** obtained Beta-Galactosidase from *Pyrococcus furiosus* enclosed in gelatin gel by cross-linking with transglutaminase. Lyophilized immobilisate was stored at 90 °C for one month without loss of activity. The immobilized enzyme catalyzed transglucosylation of 5-phenylpentanol with 10.0 equivalent of cellobiose at pH 5.0 and 70 °C for 12 h to afford 5-phenylpentyl Beta-D-Glucopyranoside in 41% yield. The immobilized enzyme was thermostable and reusable.

- **Takeomi Murata *et al.,* (2005)[30];** designed novel chromogenic substrates for endo Beta-Galactosidase on the basis of the structural features of Keratan sulfate. Kinetic analysis by this method showed that the value of V_{max}/K_m of 2 for *E. freundii* endo Beta-Galactosidase was almost equal to that of Keratan sulfate, indicating that 2 was very suitable for analytical use in an endo Beta-Galactosidase assay. Gal ß1, 4 GlcNAc ß1, 3 Gal ß1, 4 GlcNAc ß-pNP (2), which consisted two repeating units of N-acetyllactosamine was enzymatically synthesized.

- **Akolkar S. K. *et al.,* (2006)[10];** isolated *Lactobacillus acidophilus* from Eleusine coracana (fermented millet) and was characterized using several

biochemical tests. The strain was found to be homofermentative, slime forming, and Beta-Galactosidase producer. The enzyme was intracellular and various cell lysis methods was studied. Homogenization at a pressure of 2000 psig two passes showed an enzyme release of 2050 U/mL. The cell extract was purified using ultrafiltration and gel-permeation chromatography. Kinetic parameters were determined using ONPG as substrate. V_{max} and K_m was found to be 4.94 min^{-1} and 0.11 mM. The molecular weight was found to be 450-500 KDa using gel-permeation chromatography.

- **Angel Pereira Rodriguez *et al.,* (2006)[16];** constructed a hybrid protein betweeen *Kluyveromyces lactis* and *Aspergillus niger* Beta-Galactosidases that increased the yield of protein released to the growth medium. The highest levels of enzyme were obtained when the segment corresponding to the five domain of the *K. lactis* Beta-Galactosidase was replaced by the corresponding five domain of *A. niger* Beta-Galactosidase. The stability and affinity for synthetic (ONPG) or natural (lactose) substrates was higher in the hybrid than in the native state. Finally, a structural-model of the hybrid protein was obtained by homology modeling.

- **Carla Oliveira *et al.,* (2007)[50];** engineered a flocculent *Saccharomyces cerevisiae* to stably secrete *Aspergillus niger* Beta-Galactosidase in a continuous high-cell-density bioreactor. The integration of multiple copies was confirmed by genomic Southern blot analysis. Integrants with the highest Beta-Galactosidase levels had similar Beta-Galactosidase activities as a recombinant strain carrying the Beta-Galactosidase expression cassette in a YEp-based vector. This was the first study of multicopy integrant stability in a continuous bioreactor operating at different dilution rates.

- **Wutor V. C. *et al.,* (2007)[55];** determined the presence of coliforms in polluted water enzymatically by directly monitoring the activity of Beta-D-Galactosidase through the hydrolysis of yellow chromogenic substrate CPRG, which produced a red chlorophenol red (CPR) product. The objective of this study was to monitor the effect of compounds commonly found in the environment and used in water treatment on a β-GAL CPRG assay and to investigate the differences between the environmental β-GAL enzyme and the

pure commercial enzyme. Environmental β-GAL was optimally active at pH 7.8 and temperature 35 and 55 °C respectively. Environmental β-GAL behaved differently from the commercial enzyme.

3. AIM AND OBJECTIVE

The lactose-hydrolyzing enzyme, β-galactosidase (β-D-galactoside galacto hydrolase, trivially lactase) is an important enzyme used in dairy industry. β-galactosidase catalyzes the splitting of the β-galactosidic bond of lactose, which yields glucose and galactose. The monosaccharides glucose and galactose do not inherit the drawbacks of lactose such as being a pollutant in untreated whey, crystallizing out in frozen and condensed milk products, and being intolerance to many people. In some cases β-galactosidase is able to catalyze transglycosylation reactions.

The Beta-Galactosidase is an intracellular enzyme released from *Streptococcus thermophilus* and has been used to produce low lactose containing food products for lactose intolerance people and for the utilization of whey, which could otherwise be an environmental pollutant.

Streptococcus thermophilus is considered as an important organism as a starter culture of fermented products. *Streptococcus thermophilus* strain was selected for the following reasons;

a). Lactose maldigesters may consume some fermented dairy products with little or no adverse effects.

b). Lactic acid bacteria are generally regarded as safe (GRAS), so the enzyme derived from them might be used without extensive purification.

So the objective of the present study were to extract high yields of Beta-Galactosidase enzyme from *Streptococcus thermophilus* strains obtained from yoghurt samples grown in acid whey and to determine kinetic constants, to characterize and purify the enzyme by SDS-PAGE and gel-filtration chromatography.

4. PLAN OF WORK

I. Collection of samples

II. Isolation of *Streptococcus thermophilus* from Yoghurt

III. Microscopical and Biochemical characterization

IV. Production of Beta-Galactosidase enzyme grown in whey

V. Partial purification of Beta-Galactosidase enzyme by

- a. Ammonium sulphate precipitation
- b. Dialysis against 0.1M phosphate buffer (pH 7) buffer solution at 4 °C
- c. Gel-filtration chromatography using Sephadex G-100

VI. Construction of standard curve by Lowry's method

VII. Assay of Beta-Galactosidase enzyme

- a. Construction of ONP standard curve
- b. Enzymatic assay of Beta-Galactosidase

VIII. Determination of Beta-Galactosidase enzyme activity

- a. Construction of standard curve using Beta-Galactosidase

IX. Purification of Beta-Galactosidase enzyme by SDS-PAGE

X. Beta-Galactosidase confirmatory test or ONPG test

XI. Optimization of Substrate Concentration

XII. Determination of Kinetic constants

XIII. Characterization of Beta-Galactosidase enzyme

- a. Effect of pH
- b. Effect of temperature

5. MATERIALS AND INSTRUMENTS

MATERIALS USED:

S.No	Chemical Name	Company
1	Acrylamide	HI-MEDIA, MUMBAI
2	Ammonium bicarbonate	LOBA CHEMI, MUMBAI
3	Ammonium persulphate	HI-MEDIA, MUMBAI
4	Ammonium sulphate	NICE CHEMI, COCHIN
5	Ammonium citrate	HI-MEDIA, MUMBAI
6	Agar	HI-MEDIA, MUMBAI
7	Bisacrylamide	HI-MEDIA, MUMBAI
8	β-mercaptoethanol	HI-MEDIA, MUMBAI
9	Bovine Serum Albumin	HI-MEDIA, MUMBAI
10	Bromophenol blue	HI-MEDIA, MUMBAI
11	Bromocresol purple	HI-MEDIA, MUMBAI
12	Beef extract	HI-MEDIA, MUMBAI
13	β-Galactosidase	SIGMA-ALDRICH, GERMANY
14	Crystal violet	LOBA CHEMI, MUMBAI
15	Calcium chloride	SD-FINE CHEMI, MUMBAI
16	Coomassie Brilliant Blue G-250	HI-MEDIA, MUMBAI
17	Disodium hydrogen phosphate	SD-FINE CHEMI, MUMBAI
18	Glucose	RANBAXY LABORATORIES
19	Gram's iodine	LOBA CHEMI, MUMBAI
20	Glacial acetic acid	QUALIGEN, MUMBAI
21	Glycine	LOBA CHEMI, MUMBAI
22	HYA agar	HI-MEDIA, MUMBAI
23	Lactose	LOBA CHEMI, MUMBAI
24	Magnesium sulphate	LOBA CHEMI, MUMBAI
25	Mannitol	RANBAXY FINE CHEMICALS LTD., NEW DELHI
26	Methanol	LOBA CHEMI, MUMBAI
27	Nutrient agar	HI-MEDIA, MUMBAI
28	Nessler's reagent	LOBA CHEMI, MUMBAI
29	Peptone	LOBA CHEMI, MUMBAI
30	Potassium dihydrogen phosphate	SD-FINE CHEMI, MUMBAI
31	Sodium chloride	LOBA CHEMI, MUMBAI
32	Sodium hydroxide	SD-FINE CHEMI,

		MUMBAI
33	Sodium acetate	LOBA CHEMI, MUMBAI
34	Sodium carbonate	SPECTRUM REAGENTS & CHEMICALS PVT. LTD., EDAYAR, ALUVA
35	Sodium nitrate	SD-FINE CHEMI, MUMBAI
36	Sodium tartarate	LOBA CHEMI, MUMBAI
37	Saffranin	LOBA CHEMI, MUMBAI
38	SDS	SD-FINE CHEMI: MUMBAI
39	Sephadex G-100	SIGMA-ALDRICH, GERMANY
40	*Streptococcus thermophilus* (NCIM NO: 2412)	NCIM, PUNE
41	Tryptone	LOBA CHEMI, MUMBAI
42	Tris-buffer	HI-MEDIA, MUMBAI
43	Tween 80	LOBA CHEMI, MUMBAI
44	Yeast extract	LOBA CHEMI, MUMBAI

INSTRUMENTS USED

S.No	Instrument Name	Company
1	Autoclave	NEW LAB EQUIPMENT
2	Cooling Centrifuge	REMI MOTOR LTD
3	Digital Balance	SHIMADZU
4	Deep Freezer	BLUE STAR
5	Digital pH Meter	ELICO
6	Double Beam UV-Visible Spectrophotometer	ELICO
7	Electronic Digital Water Bath	GENUINE
8	Hot Air Oven	GENUINE
9	Incubator	GENUINE
10	Laminar Air Flow	GENUINE
11	Magnetic Stirrer	GENUINE
12	Refrigerator	GODREJ
13	SDS-PAGE	GENUINE

6. METHODOLOGY

1. COLLECTION OF SAMPLES[18, 19, 59]:

1. A total of 27 yoghurt samples were collected.
2. Nine samples from each brand of yoghurt namely Amul, Nilgiris, and Nestle were collected.
3. Three samples from each brand were stored at 0°C, 10 °C, and -4 °C respectively.

2. ISOLATION OF S. THERMOPHILUS FROM YOGHURT[18, 19, 21]

2.1. HYA AGAR MEDIUM: The HYA agar medium was prepared according to the composition given in Appendix 1.

2.2. PREPARATION PROCEDURE[21]

a). 20 HYA agar plates were streaked with small yoghurt sample directly from the container.

b). Another 20 HYA agar plates were streaked with small amount of yoghurt diluted with saline.

c). All the plates were incubated at 37 °C for 24 hours.

d). Colonies that were very small and white were isolated and used for further studies.

e). The small white colonies were subcultured in MRS broth and used for further studies. The composition of MRS broth was given in Appendix 2.

3. CHARACTERIZATION OF THE ISOLATED ORGANISM

3.1. MICROSCOPICAL CHARACTERIZATION OF THE ISOLATED ORGANISM:

3.1.1. SIMPLE STAINING[12]:

Procedure:

1. 2 or 3 loopfuls of a liquid culture were placed on the slide with a sterile loop.
2. Bacteria was spread on the slide.
3. The slide was passed over the flame of a burner 2 or 3 times to fix the smear.
4. The slide was placed on a staining rack.
5. The smear was covered with methylene blue and leave for 30-60 seconds.
6. The excess stain was washed off with distilled water from the wash bottle. The water was let run down the tilted slide.

7. The smear was gently blotted with a paper towel or absorbent paper and left to dry.
8. The stained smears were examined microscopically using the low, high-dry and oil immersion objectives.

3.1.2. GRAM STAINING[12]:

Procedure:

1. 2 or 3 loopfuls of a liquid culture were placed on the slide with a sterile loop.
2. The bacteria was spread on the slide.
3. The slide was passed over the flame of a burner 2 or 3 times to fix the smear.
4. The slide was placed on a staining rack.
5. The smear was covered with crystal violet for 30 seconds.
6. The crystal violet was washed off with water by squirting the water, so it runs through the smear.
7. The smear was covered with Gram's iodine for 10 seconds.
8. The smear was gently washed with water and decolourized with alcohol and the alcohol was washed off.
9. The smear was covered with saffranin for 30 seconds.
10. The smear was washed with water and blot dried.
11. The stained smear were examined microscopically using the low, high-dry and oil immersion objectives.

3.1.3. MOTILITY TESTING[12]:

Hanging Drop Method:

1. A depression slide was obtained.
2. A cover slip was picked up carefully, the petroleum jelly was scraped with an edge of the cover slip to get a small ring of petroleum jelly and repeated with other three edges keeping the petroleum jelly on the same side of the cover slip.
3. The cover slip was placed on a paper towel with the petroleum jelly side up.
4. A drop of microbial suspension in the center of the cover slip was placed. The depression slide was placed on the cover slip and quickly inverted so the drop was suspended.
5. The slide was examined under low power by locating the edge of the drop and moving the slide so the edge of the drop crossed the center of the field.
6. The light was reduced with iris diaphragm and focus. The different sizes, shapes, and types of movements were observed.

In microbiological staining, the organism was compared with standard strain of *Streptococcus thermophilus* (NCIM NO: 2412) obtained from NCBI, Pune.

3.2. BIOCHEMICAL CHARACTERIZATION OF THE ISOLATED ORGANISM[21, 35]:

3.2.1. CATALASE ACTIVITY:

Most microorganisms grown aerobically possessed the enzyme catalase. The lactic acid bacteria do not normally produce a detectable amount of catalase. The isolated strains were checked for the production of the enzyme catalase. A loopful of organism subcultured in MRS broth was placed on the slide, 1 ml of hydrogen peroxide solution was added to test whether gas bubbles form or not. No formation of free oxygen bubbles indicated the absence of catalase.

3.2.2. FERMENTATION OF SUGARS:

i). Fermentation of Lactose:

Lactose broth was prepared and inoculated with the organism and incubated at 37 °C for 24 hours. The change in colour to yellow indicated the fermentation of lactose. The composition of lactose broth was given in Appendix 3.

ii). Fermentation of Glucose:

Glucose broth was prepared and inoculated with the organism and incubated at 37 °C for 24 hours. The change in colour to yellow indicated the fermentation of glucose. The composition of glucose broth was given in Appendix 4.

iii). Fermentation of Mannitol:

Mannitol broth was prepared and inoculated with the organism and incubated at 37 °C for 24 hours. No change in colour indicated that mannitol fermentation was negative. The composition of mannitol broth was given in Appendix 3.

3.3. Identification test for *Streptococcus thermophilus*[22]:

The isolates were streaked on the ST agar plates, incubated at 37°C for 24 h and observed. The cultures of *S. thermophilus* formed well-developed, yellow colonies on ST agar plates within 24 h of incubation. The composition of ST agar was given in Appendix 6. Thus, ST agar could be used for the enumeration of *S. thermophilus* from yoghurt containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, provided that the plates were incubated aerobically at 37°C for 24 h.

4. PRODUCTION OF BETA-GALACTOSIDASE ENZYME GROWN IN WHEY

4.1. PREPARATION OF CELL FREE EXTRACTS[5, 6, 7, 8, 9]:

1. Acid whey was obtained from MMD Dairy Institute, Erode.
2. The whey was deproteinized by heating at 90 °C (pH 4.5) for 10 minutes.
3. Then it was filtered through Whatman No:1 filter paper to remove coagulated protein and adjusted to pH 7.0.
4. It was sterilized at 121 °C for 15 minutes.
5. The sterile whey was inoculated with 1% active culture of the isolated organisms and incubated at 40 °C for 24 hours.
6. At the end of incubation, cells were harvested by centrifuging at 15,000 rpm for 20 minutes.
7. The pellets were washed twice with 0.1 M phosphate buffer (pH 7.0) solution.
8. The pellets stored in 0.1 M phosphate buffer were added to sterilized sand and triturated with addition of buffer for an hour and was centrifuged.
9. To the supernatant, ammonium sulphate was added to give 70% saturation with constant stirring (ammonium sulphate precipitation method).
10. The precipitated protein were again centrifuged and the pellets were stored in 0.1 M phosphate buffer and purified by using dialysis membrane.

5. PARTIAL PURIFICATION OF BETA-GALACTOSIDASE

5.1. AMMONIUM SULPHATE PRECIPITATION[20]:

The crude enzyme prepared was brought to 70% saturation with ammonium sulphate with continuous stirring in a magnetic stirrer and kept undisturbed for 1 hr at 4° C and centrifuged at 8000 rpm for 20 min at 4 °C. Then the precipitate was collected and stored at 4 °C for further purification.

5.2. DIALYSIS[5, 20]:

Procedure:

1. Individual solution containing fraction of precipitate from 70% saturation of ammonium sulphate was dialyzed against 0.1 M phosphate buffer (pH 7) solution.
2. A pretreated dialysis membrane was taken, at one end it was clamped tightly from the open end the fraction precipitate solution was added into the dialysis membrane.

3. Then open end of the dialysis membrane bag was closed tightly using clamps.
4. The closed dialysis bag was kept in the beaker containing 0.1 M phosphate buffer (pH 7) solution.
5. The buffer was stirred gently with magnetic stirrer to improve the solute exchange.
6. The buffer was then tested for ammonium sulphate every 1 hour initially and then half an hour towards the end using Nessler's reagent, which gave brown colour with ammonium sulphate. The buffer was changed continuously by exchanging with fresh buffer solution.
7. After 7-8 hours, the equilibrium was achieved, when white precipitate was obtained.
8. The dialyzed bag was removed and the partially purified enzyme was used for the further study of enzyme and stored at refrigerated temperature.

5.3. GEL FILTRATION CHROMATOGRAPHY[24]:

Procedure:

1. 2 gm Sephadex G-100 powder was weighed and swelled in a 200 ml 0.1 M phosphate buffer (pH 7) solution for 72 hours at 20 °C.
2. To pack a column, the excess buffer in the gel matrix was decanted and fairly thick slurry was used.
3. The column was filled one-fourth with buffer and the outlet was opened, when the flow through the outlet was normal (without any air bubble) the flow was stopped.
4. Now the gel slurry was poured slowly through the sides using a funnel.
5. As material settled down to 5-6 cm by gravity, the outlet was opened slowly so that drops could form and then fall, due to this gel settled faster.
6. Once the column was packed without air bubbles then the column was stabilized by passing 2-3 bed volume of buffer solution.
7. Then 1 ml of protein sample was added in column and the protein was allowed to touch the Sephadex gel to maintain as wet gel surface, to prevent drying and cracking of column.
8. The flow rate was adjusted 3 ml per hour or one drop per min, totally 25 fractions was collected, absorbance measured at 280 nm using UV-spectrophotometer.
9. Finally graph was plotted between Fraction number Vs Absorbance, which was shown in Results and Discussion.

10. Fractions which gave peak were used for further studies.

6. CONSTRUCTION OF STANDARD CURVE BY LOWRY'S METHOD

6.1. Preparation of standard curve of protein (BSA) by Lowry's method[23]:

i) Preparation of Solution A:

0.5 gm of copper sulphate and 1 gm of sodium tartarate was dissolved in small amount of distilled water and made to the volume 100 ml with the same solution was made homogenious and was kept at room temperature (28 °C).

ii) Preparation of Solution B:

20 gm of sodium bicarbonate and 4 gm of sodium hydroxide was taken in 1000 ml volumetric flask and dissolved in small amount of distilled water, then volume was made upto 1000 ml with distilled water.

iii) Preparation of Solution C:

1 ml of Sol A and 50 ml of Sol B was taken in a 100 ml beaker and the solution was mixed thoroughly.

iv) Preparation of Solution D:

10 ml of folin cocatteau reagent was taken in a beaker, to this 10 ml of distilled water was added and the solution was mixed thoroughly.

v) Preparation of stock solution of standard protein (BSA) solution:

10 mg of Bovine Serum Albumin (BSA) was dissolved in small quantity of distilled water and volume was made upto 10 ml by distilled water, which gave concentration of 1000 µg/ml.

vi) Preparation of working standard:

1 ml of stock solution was taken in 10 ml volumetric flask, to it distilled water was added upto the mark and this gave a concentration of 100 µg/ml.

6.2. Procedure:

1. Six test tubes were taken and marked as a, b, c, d, e, and f.
2. 0.2 to 1 ml of working standard solution was taken in test tubes marked a, b, c, d, e, and f respectively.
3. Volume was made upto 1 ml by distilled water for each tube, thus gave concentration from 20 to 100 µg/ml.

4. 1 ml of distilled water was taken in test tube marked ‘f’ and it was taken as blank.
5. 2.5 ml of solution C was added into each test tube from a to f.
6. 1 ml of solution D was added to each test tube.
7. The above solutions were vortexed, wrapped in black sheet and incubated for 30 min.
8. After 30 min of incubation the absorbance was measured at 660 nm using double beam spectrophotometer.
9. A graph of absorbance Vs concentration was plotted.

This standard calibration curve of protein was used for estimation of protein content and hence for the specific activity of enzyme.

7. ASSAY OF BETA-GALACTOSIDASE ENZYME

7.1. Preparation of ONP standard curve[11]

7.1.1. Preparation of ONP solution for standard curve construction:

ONP was an important compound as an indicator for Beta-Galactosidase activity. In this assay, free ONP at different dilutions level were used to construct ONP standard curve. During construction of ONP standard curve, known amount of ONP with several dilutions were prepared. Each dilution gave a specific yellow color under the assay conditions with absorption peak at 420 nm.

ONP (0.033384 g) was mixed with 1 ml 0.05 M phosphate buffer (at pH 7) and 3 ml ethanol. Final volume of the mixture was set to 4 ml. This mixture (4 ml) was agitated until most of the solid particles were become soluble. This mixture was transferred into preheated water bath at 45 °C. Melting temperature of ONP in water is 44 °C- 45 °C. This mixture was taken as ONP (60 mM) stock solution. The several dilutions of stock solution were carried out by 0.05 M sodium phosphate buffer at pH 7. Each dilution from the stock solution gave a specific yellow color under the assay conditions with absorption peak at 420 nm.

Blank solution was prepared by mixing 3 ml alcohol and 1ml sodium phosphate buffer (0.05 M at pH 7). For each set of dilutions of stock solution, blank solution was also diluted. Absorption values were read against the blank solutions in UV spectrophotometer at 420 nm. A graph was plotted between concentration of ONP and absorbance values.

Table for Construction of ONP Standard Curve:

S.No.	Volume of Stock Solution using ONP (ml)	Buffer [0.05 M Sodium Phosphate Buffer] (ml)	Final Volume of ONP solution (ml)	Concentration of ONP (mM)
1	0.5	4.5	5	6
2	0.5	2	2.5	1.2
3	1	9	10	0.6
4	3	3	6	0.3
5	3	3	6	0.15
6	1	4	5	0.12
7	3	4.5	7.5	0.06
8	1	4	5	0.012
9	0.5	4.5	5	0.006

7.2. Enzymatic assay of ß-Galactosidase[26, 27]

7.2.1. Principle:

Beta-Galactosidase hydrolyzes o-nitrophenyl ß-D-Galactopyranoside (ONPG) into o-nitrophenol and ß-D-Galactose.

ONP ß-D-Galactopyranoside + H_2O ----------> o-nitrophenol + ß-D-Galactose

7.2.2. Reagents Used:

1. Reagent A: 100 mM Sodium Acetate Buffer (pH 6.0 at 37 °C):
2. Reagent B: 2.0 mM ONPG with 0.01% (w/v) Bovine Serum Albumin, pH 6.0, (substrate solution):
3. Reagent C: 1000 mM Sodium Carbonate Solution:

7.2.3. Procedure:

The following Reagent B (substrate solution) and deionized water were pippeted, mixed by inversion and equilibrated to 37 °C. Then the enzyme solution was added, immediately mixed by inversion and incubated for 10 minutes. After this Reagent C was added, mixed by inversion and the absorbance was recorded at 420 nm for both the test and blank solutions.

S.No.	Reagents	Test (ml)	Blank (ml)
1	Reagent B	0.50	0.50
2	Deionized water	0.30	0.30
3	Enzyme solution	0.20	-
4	Reagent C	4.00	4.00

Unit Definition:

One unit will hydrolyze 1.0 micromole of o-nitrophenyl ß-D-Galactopyranoside (ONPG) into o-nitrophenol and ß-D-Galactose per minute at pH 6.0 at 37 °C.

8. DETERMINATION OF BETA-GALACTOSIDASE ACTIVITY

8.1. Preparation of Standard Curve[28]

1. To prepare standard curve, standard Beta-Galactosidase between 0 and 6.0 units were used.
2. The dilution series was prepared in 1X Reporter Lysis buffer immediately before use.
3. 10 µl of 1 U/µl Beta-Galactosidase to 990 µl of 1X Reporter Lysis buffer was added, and vortexed. 10 µl of this 1:100 dilution to 990 µl of 1X Reporter Lysis buffer was added, and vortexed to make 1:10,000 stock solution.
4. For 0 µl of stock solution, 150 µl of 1X Reporter Lysis buffer was taken; for 10 µl of stock solution, 140 µl of 1X Reporter Lysis buffer was taken; for 20 µl of stock solution, 130 µl of 1X Reporter Lysis buffer was taken. The test tubes were prepared upto 60µl of stock solution.
5. 150 µl of Assay 2X buffer was added to each of the tubes. All samples were mixed by vortexing briefly.
6. The tubes were incubated at 37 °C for 30 minutes or until a faint yellow colour had developed. Colour development continued for approximately 3 hours.
7. The reactions were stopped by adding 500 µl of 1 M sodium carbonate and mixed by vortexing briefly. The absorbance was read at 420 nm immediately after addition of 1 M sodium carbonate.

8. The graph was plotted for the absorbance Vs concentration of standard Beta-Galactosidase.
9. The sample (S1) enzyme solution was taken instead of standard enzyme and the steps 3-7 were followed and the units of enzyme were determined.

Table for Construction of Standard Curve:

S.No.	Beta-Galactosidase Standard (milliunits)	Volume of Stock solution (µl)	Volume of 1X Reporter Lysis Buffer (µl)
1	0	0	150
2	1	10	140
3	2	20	130
4	3	30	120
5	4	40	110
6	5	50	100
7	6	60	90

9. PURIFICATION OF BETA-GALACTOSIDASE ENZYME BY SDS PAGE[25]:

9.1. Preparation of solution for SDS-PAGE

1. 30% Acrylamide mix solution

7.5 gm of acrylamide and 0.2 gm of bisacrylamide was added in 25 ml of volumetric flask and 15 ml of distilled water was added, these chemicals was dissolved by heating at 37 °C, then volume was made upto 25 ml by distilled water, it was then filtered through nitrocellular filter and was kept in amber colour bottle at room temperature.

2. 1.5 M tris (pH 8.8)

18.16 gm of tris base was dissolved in some amount of distilled water and volume was made upto 100 ml by distilled water.

3. 1.0 M tris (pH 6.8)

12.11 gm of tris base was dissolved in some amount of distilled water and volume was made upto 100 ml by distilled water.

4. 10% SDS (pH 7.2)

5 gm of sodium dodecyl sulphate was taken in 50 ml volumetric flask and dissolved in distilled water by heating at 40 °C and then volume was made upto 50 ml by distilled water.

5. 10% Ammonium persulphate

1 gm of ammonium persulphate was taken in 10 ml volumetric flask, dissolved in distilled water and volume was made upto 10 ml by distilled water.

6. Methanol : acetic acid solution

250 ml of methanol and 200 ml of distilled water was mixed and was added in 500 ml volumetric flask. To this solution, 50 ml of glacial acetic acid was added.

7. Preparation of staining reagent

1.25 ml of Coomasie Brilliant Blue R-250 was mixed with 500 ml methanol acetic acid solution.

8. Preparation of destaining solution

250 ml of methanol and 200 ml of distilled water were mixed and added to 500 ml volumetric flask. To this solution 50 ml of glacial acetic acid was added.

9. Preparation of 2 X SDS gel – loading buffer (10 ml)

In 10 ml volumetric flask 0.4 gm of SDS, 25 µl of bromophenol blue + 2 ml of glycerol + 0.121 gm tris – HCl and 1 ml of 2-mercaptophenol was added and volume was made upto 10 ml by distilled water and mixed thoroughly.

10. Preparation of tris glycine electrophoresis buffer (pH 8.2-8.4)

In 500 ml volumetric flask 1.51 gm of tris base + 9.4 gm of glycine was added and dissolved in 200 ml of distilled water. Then 10 ml of 10 % SDS solution was added, mixed and volume was made upto 500 ml with distilled water.

9.2. Preparation of resolving gel (15%)

For 40 ml resolving gel (15%) following solutions was added

Reagents	**Volume**
Water	9.2 ml
30% acrylamide mixture	20 ml
1.5 M tris (pH 8.8)	10 ml
10% SDS	0.4 ml
10% ammonium persulphate	0.4 ml

TEMED	0.016 ml

9.3. Preparation of stacking gel (5%)

For 10 ml stacking gel (5%) following solutions was added

Reagents	Volume
Water	6.75 ml
30% acrylamide mixture	1.675 ml
1.0 M tris (pH 6.8)	1.25 ml
10% SDS	0.1 ml
10% ammonium persulphate	0.1 ml
TEMED	0.01 ml

9.4. Preparation of sample

25 µl of enzyme solution was added to 15 µl of 1 x SDS gel loading buffer and the mixture was heated at 100 °C for 3 minutes for dissociation of proteins.

9.5. PROCEDURE:

9.5.1. Pouring of SDS – Polyacrylamide Gels

1. The glass plates were cleaned and assembled with the help of strips and clips.
2. The acrylamide solution (resolving gel) was poured into the gap between the glass plates, by keeping the sufficient space for the stacking gel.
3. With the help of Pasteur pipette n-butanol was carefully overlayed. The gel was kept in vertical position at room temperature (28 °C).
4. After 30 min (polymerization completed), overlay of n-butanol was poured off. The top of the gel was washed with water several times to remove any unpolymerized acrylamide.
5. The fluid drained from the top of the gel and remaining water was removed with the edge of a paper towel.
6. The freshly prepared stacking gel solution was poured directly onto the surface of the polymerized resolving gel.
7. Immediately insert a clean Teflon comb into the stacking gel solution. Then again some stacking gel solution was added to fill the space of the comb completely. The gel was placed in a vertical position for 30 min at room temperature.

9.5.2. Running the gel

1. After completion of polymerization, the Teflon comb was removed carefully.
2. The gel was mounted in the electrophoresis apparatus.
3. The tris-glycine electrophoresis buffer was added to the top and bottom reservoir.
4. With the help of bent hypodermic needle attached to a syringe air bubbles was removed which was trapped at the bottom of the gel between the glass plates.
5. The 15 µl of enzyme solution (crude) was loaded into the bottom of the wells with the help of micro liter syringe (micropipette), the equal volume of enzyme solution [70% saturation] and enzyme solution purified by gel filtration chromatography was loaded into the bottom of the wells.
6. The electrophoresis apparatus was attached to an electric power supply (+ve electrode connected to bottom buffer reservoir and –ve electrode connected to upper buffer reservoir.
7. Initially 100 V was applied to the gel and then it was increased to 150 V, when the dye front was moved into the resolving gel, the gel was run until the bromophenol blue reaches the bottom of the resolving gel (4 h). Then the power supply was turned off.
8. The glass plates were removed carefully from the electrophoresis apparatus and were placed on a paper towel.
9. The glass plates were separated apart.
10. For marking the orientation of the gel, the corner of the bottom of gel which was closest to the left most well was cut.

9.5.3. Staining of the SDS – Polyacrylamide Gel with Coomasie Brilliant Blue:

1, The gel was immersed in the tray containing 250 ml of staining solution.
2. The tray was rotated slowly upto one hour.
3. After 1 hour, the staining solution was removed, then the gel was soaked in the methanol:acetic acid solution without the dye, it was also rotated slowly upto 4 hours for destaining the gel. The destaining solution was changed two times.
4. After destaining the gel, the gel was kept in the white illuminator for observing the bands and for photograph.

10. ONPG-CONFIRMATORY TEST FOR BETA-GALACTOSIDASE[29]:

Lactose utilization requires a couple of enzymes, one of which was Beta-Galactosidase. In this test, a molecular decoy called ONPG (Ortho-nitrophenyl-β-D-galactopyranoside) was used that will turn to a yellow color in the presence of this enzyme. Since this enzyme was made only in the presence of the lactose substrate, it is necessary to grow this organism on media high in lactose.

10.1. PROCEDURE:

1. The isolated organism was grown in a medium rich in lactose to induce the production of beta-galactosidase enzyme.
2. 0.5 ml of the saline (0.85-0.9% NaCl) was pipetted into a sterile tube.
3. The organism was inoculated and the ONPG discs were added in a sterile manner to the tube.
4. The tube was incubated at 37 °C for 4 hours.
5. The disc was observed for colour change.

11. OPTIMIZATION OF SUBSTRATE CONCENTRATION

11.1. Optimization of Substrate Concentration[11, 30, 40]:

The enzyme solution was used to observe the effect of substrate concentration on β-galactosidase activity. Kinetic constants, K_m and V_{max}, of the β-galactosidase were determined by changing the ONPG substrate concentration. ONPG substrate concentrations of 1 to 35 mM were taken (in 0.1 M phosphate buffer at pH 7). Michaelis-Menten plot and Lineweaver-Burk double reciprocal plot were constructed to calculate the K_m and V_{max}.

12. DETERMINATION OF KINETIC CONSTANTS

12.1. Determination of K_m and V_{max} Value[11, 30, 40]:

For the determination of K_m and V_{max} values, Lineweaver-Burk double reciprocal plot was used. A graph of $1/V_o$ versus $1/[S]$ was plotted to yield a straight line with an intercept on the Y-axis, that is, $1/V_{max}$ a slope = K_m/V_{max} and intercept on the negative side of the X-axis.

13. CHARACTERIZATION OF BETA-GALACTOSIDASE ENZYME

13.1. EFFECT OF pH

13.1.1 Determination of Optimum pH[5, 11]:

To determine the optimum pH, the β- galactosidase activity was studied within the range of pH from 4 to 8. Three different buffers were used; 0.05 M citrate buffer was used for pH range between 4 and 6.2, 0.05 M sodium phosphate buffer was used for pH range between 6.2 and 8, and 0.05 M tris-HCl buffer was used for pH 8. The assay was carried out using the above mentioned protocol given in 7.2. Enzyme activity was calculated.

13.2. EFFECT OF TEMPERATURE

13.2.1. Determination of Optimum Temperature[5, 11]:

To determine optimum temperature, the β-galactosidase activity of enzyme was measured at different temperatures 15 to 55 °C at optimum pH 7.2. The assay was carried out using the above mentioned protocol given in 7.2. Enzyme activity was calculated.

7. RESULTS AND DISCUSSION

1. COLLECTION OF SAMPLES

Commercial brand yoghurts were collected and stored at different temperatures. Nine samples from each brand of yoghurt namely, Amul (A1, A2, A3, A4, A5, A6, A7, A8, A9), Nilgiris (N1, N2, N3, N4, N5, N6, N7, N8, N9), and Nestle (NE1, NE2, NE3, NE4, NE5, NE6, NE7, NE8, NE9) were collected. The samples A1, A2, A3, N1, N2, N3, NE1, NE2, NE3 were stored at 0 °C, samples A4, A5, A6, N4, N5, N6, NE4, NE5, NE6 were stored at 10 °C, and samples A7, A8, A9, N7, N8, N9, NE7, NE8, NE9 were stored at -4 °C.

2. ISOLATION OF *S. THERMOPHILUS* FROM YOGHURT

The HYA agar medium was prepared and 20 plates were streaked with small yoghurt sample directly from the container. Another 20 HYA agar plates were streaked with small amount of yoghurt diluted with saline, incubated at 37 °C for 24 hours. Colonies which were very small and white were isolated from 27 samples.

From Amul, the samples A1, A4, A5, and A6 showed clear white colonies and were isolated and subcultured in MRS broth for further studies. From Nilgiris, the samples N5 and N6 showed clear white colonies and were isolated and subcultured in MRS broth for further studies. From Nestle, the samples NE4 and NE5 showed clear white colonies and were isolated and subcultured in MRS broth for further studies.

3. CHARACTERIZATION OF THE ISOLATED ORGANISM

3.1. MORPHOLOGICAL CHARACTERIZATION

Microscopical identification of the sample isolates were done by Simple staining, Gram staining, and Motility testing.

The five sample isolates A4, A5, A6, N6, and NE5 were identified as spherical or cocci shaped, gram-positive, and non-motile, having characteristics of *Streptococcus* species. Three sample isolates A1, N5, and NE4 were identified as rod shaped, gram-positive, and non-motile, having characteristics of *Lactobacillus* species. The results of Microscopical identification of the sample isolates were shown in table no. 1.

3.2. Biochemical Characterization

Biochemical characterization was done for the sample isolates A4, A5, A6, N6, and NE5, which were identified as spherical shaped, gram-positive, and non-motile, having characteristics of *Streptococcus* species. The following tests; a). Catalase test b). Glucose fermentation test c). Lactose fermentation test d). Mannitol fermentation test and e). ST agar test were done.

The five sample isolates A4, A5, A6, N6, and NE5 gave negative results for catalase test and mannitol fermentation test and positive results for glucose fermentation test, lactose fermentation test, and ST agar test. *L. delbrueckii ssp. bulgaricus* either did not grow or formed tiny, white, cottony colonies that could easily be distinguished from *S. thermophilus*. ST agar test was the identification test for *Streptococcus thermophilus*, so the five isolates A4, A5, A6, N6, and NE5 were subcultured in MRS broth and used for further studies. The results of biochemical characterization were shown in table no. 2

4. PRODUCTION OF BETA-GALACTOSIDASE ENZYME GROWN IN WHEY

4.1. PREPARATION OF CELL FREE EXTRACTS

Acid whey was obtained, deproteinized by heating, filtered through Whatman filter paper and sterilized. The sterile whey was inoculated with the culture of the five sample isolates A4, A5, A6, N6, and NE5 and incubated at 40 °C for 24 h.

At the end of incubation, cells were harvested by centrifugation. To the supernatant, ammonium sulphate was added to give 70% saturation with constant stirring in a magnetic stirrer. All the five sample isolates examined synthesized beta-galactosidase with yields ranging from 5.07 to 8.36 U/ml. But the sample isolate A5 was selected for further studies because of high productivity (7.76 U/ml).

S.No.	Sample isolates	Yield (U/ml)
1	A4	6.25
2	A5	7.76
3	A6	6.86
4	N6	5.98
5	NE5	5.37

5. PARTIAL PURIFICATION OF BETA-GALACTOSIDASE

5.1. AMMONIUM SULPHATE PRECIPITATION

Beta-Galactosidase was precipitated by ammonium sulphate at 70 % saturation and kept undisturbed for 1 hr at 4° C and centrifuged at 8000 rpm for 20 min at 4 °C. Then the precipitate was collected and stored at 4 °C for further purification.

5.2. DIALYSIS

The precipitate dissolved in 0.1 M phosphate buffer was transferred to a pretreated dialysis membrane. Then the dialysis membrane was resuspended in a minimal volume of 0.1 M phosphate buffer and dialysed overnight against the same buffer.

5.3. GEL FILTRATION CHROMATOGRAPHY

The enzyme solution obtained after dialysis was applied to the top of a column containing Sephadex G-100 slurry equilibrated with 0.1 M phosphate buffer and then eluted with the same buffer. The absorbance was read at 280 nm. The fractions 5, 14, 15, 20 gave high peaks. In these, peaks 14, 15 showed enzyme activity. So these fractions were pooled and used for further studies. The results of gel filtration chromatography were shown in table no. 3 and fig. no. 3.

6. CONSTRUCTION OF STANDARD CURVE BY LOWRY'S METHOD

The standard curve for protein estimation by Lowry's method were constructed using various concentrations of BSA as a standard.

From the standard curve, the protein concentration of the sample isolate A5 was found to be 67 µg/ml. The results of estimation of protein were shown in table no. 4 and fig. no. 4.

7. ASSAY OF BETA-GALACTOSIDASE ENZYME

7.1. Preparation of ONP standard curve

The standard curve for ß-Galactosidase was constructed using various concentrations of o-nitrophenol (ONP) as standard solution. The graph was plotted between concentrations of ONP against absorbance. The results of the standard curve preparation for ONP were shown in table no. 5 and fig. no. 5.

7.2. Enzymatic assay of ß-Galactosidase

The enzymatic assay of ß-Galactosidase was done by using construction of ONP standard curve. From the standard curve the activity of ß-Galactosidase was found to be 7.76 U/ml.

8. DETERMINATION OF BETA-GALACTOSIDASE ACTIVITY

8.1. Preparation of Standard Curve

The standard curve was constructed by using 1X PBS buffer, Assay 2X buffer, and 1 M sodium carbonate. From the standard curve, the units of enzyme for the isolate A5 was found to be 3.6 milliunits. The results for the construction of standard curve using standard Beta-Galactosidase enzyme were shown in table no. 6 and fig. no. 6.

9. PURIFICATION OF BETA-GALACTOSIDASE ENZYME BY SDS PAGE

The purification of ß-Galactosidase by SDS-PAGE was done using resolving gel and stacking gel. The enzyme solution with the loading dye was added into the wells and electrophoresis was started at 50 V and after ½ an hour it was changed to 100 V.

After the dye reached the bottom of the gel, the power was turned off and the gel was removed from the plates and immersed in Coomasie staining solution overnight. During this time period the protein absorbed Coomasie blue. Then the gel was transferred into destaining solution. Finally the gel was observed in white illuminator for bands. A single band was observed. The results for purification of ß-Galactosidase by SDS-PAGE were shown in fig. no. 7.

10. ONPG-CONFIRMATORY TEST FOR BETA-GALACTOSIDASE

The isolated organism was grown in a medium rich in lactose to induce the production of beta-galactosidase enzyme. To a sterile tube, saline was added, the organism was inoculated and ONPG discs were added. The disc was observed for colour change. The disc changed to yellow colour, which indicates the presence of Beta-Galactosidase enzyme. The results for the ONPG confirmatory test were shown in fig. no. 8.

11. OPTIMIZATION OF SUBSTRATE CONCENTRATION

For the determination of optimum substrate concentration for partially purified Beta-Galactosidase from the isolated organism, the substrate ONPG was added in the concentration range from 3 mM to 33 mM.

As the substrate concentration was increased above 3 mM rate of reaction also increased progressively up to 24 mM. When the concentration was increased above 24 mM the rate of reaction remained constant. This showed that Beta-Galactosidase obtained from *S. thermophilus* followed the Michaelis-menten equation.

Hence, optimum substrate concentration was found to be 24 mM. Justifying that, at this concentration enzyme remained saturated with substrate for 10 min and rate of reaction was maximum at this concentration. The results of optimization of substrate concentration for Beta-Galactosidase activity were shown in table no. 7 and fig. no. 9.

12. DETERMINATION OF KINETIC CONSTANTS

K_m was numerically equivalent to the substrate concentration that yields half maximal velocity. The numerical value of K_m was very important for the following reasons.

a). If substrate in the reaction medium is too low than K_m the rate of the enzyme would be very sensitive to changes in the substrate amount.

b). Since K_m was a constant for a given enzyme, its numerical value provides a means of comparing enzymes from different organisms or the same organisms at different stages of development.

c). By measuring the effects of different compounds on K_m, important activators and inhibitors might be identified.

The K_m and V_{max} values approximately were found to be 3.05 mM and 2.8 U/ml respectively.

The linearity of Lineweaver-Burk double reciprocal plot suggested that the isolated Beta-Galactosidase followed Michaelis-Menten kinetics. The results for the determination of the kinetic constants were shown in table no. 8 and fig. no. 10.

13. CHARACTERIZATION OF BETA-GALACTOSIDASE ENZYME

13.1. EFFECT OF pH

The graph showed that enzymatic activity increases with increase in pH from 5 to 7, at the pH 7.2 the enzymatic activity was maximum and was found to be at 2.79 U/ml. After pH 7.2, the enzymatic activity decreased and at pH 8 it was destroyed drastically.

Hence, optimum pH was found to be 7.2. It means that at pH 7.2, enzyme remains in its native confirmation. The groups in the active site of Beta-Galactosidase are available in the dissociated form for reaction with the substrate. So, further study was carried out at pH 7.2. The results of assaying the sample enzyme at various pH levels were shown in table no. 9 and fig. no. 11.

13.2. EFFECT OF TEMPERATURE

As the temperature increased from 15 °C to 40 °C, the enzyme activity also increased progressively. Above 40 °C, enzyme activity decreased gradually up to 55 °C.

Thus the optimum temperature for enzyme activity was found to be 40 °C. After this, the enzymes being protein in nature, got denatured and assumed that the tertiary structure of the enzyme would be disrupted and the rate of enzyme action was decreased. The tertiary structure of an enzyme was maintained primarily by a large number of weak non-covalent bonds. The results for the determination of optimum temperature for Beta-Galactosidase activity were shown in table no. 10 and fig. no. 12.

Table No. 1: Morphological Characterization

S. No.	Sample isolates	Simple staining	Gram Staining	Motility test
1	A1	Rod	+	-
2	A4	Spherical	+	-
3	A5	Spherical	+	-
4	A6	Spherical	+	-
5	N5	Rod	+	-
6	N6	Spherical	+	-
7	NE4	Spherical	+	-
8	NE5	Rod	+	-

Table No. 2: Biochemical Characterization

S.No.	Name of the test	Sample isolates				
		A4	A5	A6	N6	NE4
1	Catalase	-	-	-	-	-
2	Glucose fermentation	+	+	+	+	+
3	Lactose fermentation	+	+	+	+	+
4	Mannitol fermentation	-	-	-	-	-
5	ST agar test	+	+	+	+	+

Table No. 3: Gel Filtration Chromatography

S.No.	Fraction No.	Absorbance at 280 nm
1	1	0.001
2	2	0.002
3	3	0.012
4	4	0.013
5	5	0.178
6	6	0.038
7	7	0.038
8	8	0.039
9	9	0.029
10	10	0.029
11	11	0.052
12	12	0.043
13	13	0.045
14	14	0.821
15	15	0.449
16	16	0.071
17	17	0.085
18	18	0.093
19	19	0.071
20	20	0.153
21	21	0.047
22	22	0.048
23	23	0.039
24	24	0.039
25	25	0.038

Table No. 4: Standard Curve for BSA

S.No	Concentration (µg/ml)	Absorbance at 660 nm
1	0	0.000
2	20	0.199
3	40	0.391
4	60	0.610
5	80	0.832
6	100	1.011
7	A5	0.691

Table No. 5: ONP Standard Curve

S.No	Concentration of o-nitrophenol (mM)	Absorbance at 420 nm
1	0	0.000
2	1.2	1.312
3	0.6	0.731
4	0.3	0.374
5	0.15	0.192
6	0.12	0.153
7	0.06	0.057
8	0.012	0.019
9	0.006	0.013
10	A5	0.621

Table No. 6: Construction of Standard Curve using Beta-Galactosidase

S.No	β-Gal (milliunits)	Absorbance at 420 nm
1	0	0.000
2	1	0.181
3	2	0.432
4	3	0.623
5	4	0.811
6	5	1.021
7	6	1.184
8	A5	0.711

Table No 7: Optimization of Substrate Concentration

S.No.	Substrate Concentration (mM)	Activity (U/ml)
1	0	0.000
2	1	1.023
3	3	1.491
4	5	1.783
5	7	1.972
6	9	2.155
7	10	2.234
8	13	2.416
9	15	2.513
10	17	2.587
11	19	2.652
12	21	2.711
13	23	2.756
14	25	2.769
15	27	2.794
16	29	2.802
17	31	2.811
18	33	2.815
19	35	2.817

Table No. 8: Determination of K_m and V_{max}:

S.No.	1/[S]	1/[V]
1	0	0.000
2	1	0.977
3	0.33	0.691
4	0.20	0.561
5	0.14	0.507
6	0.11	0.464
7	0.09	0.448
8	0.08	0.414
9	0.06	0.398
10	0.06	0.386
11	0.05	0.377
12	0.05	0.369
13	0.04	0.363
14	0.04	0.361
15	0.04	0.358
16	0.03	0.357
17	0.03	0.356
18	0.03	0.355
19	0.03	0.355

Table No. 9: Effect of pH

S.No.	pH	Activity (U/ml)
1	5	1.523
2	5.2	1.551
3	5.4	1.594
4	5.6	1.612
5	5.8	1.641
6	6	1.585
7	6.2	1.586
8	6.4	1.735
9	6.6	1.891
10	6.8	2.012
11	7	2.568
12	7.2	2.791

13	7.4	2.495
14	7.6	2.367
15	7.8	1.698
16	8	1.479

Table No. 10: Effect of temperature

S.No.	Temperature (℃)	Activity (U/ml)
1	15	1.654
2	20	2.478
3	25	3.214
4	30	4.012
5	35	4.713
6	40	4.985
7	45	3.756
8	50	2.967
9	55	2.312

Figure No. 1: HYA agar medium with white colonies.

1a). White colonies after streaking yoghurt directly from the container

1b). White colonies after streaking yoghurt mixed with saline

1c). White colonies after streaking yoghurt mixed with saline

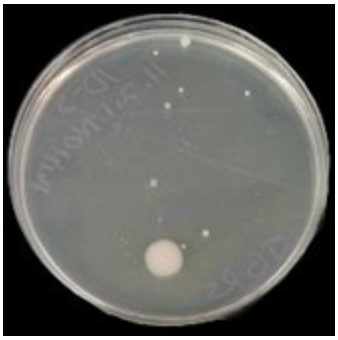
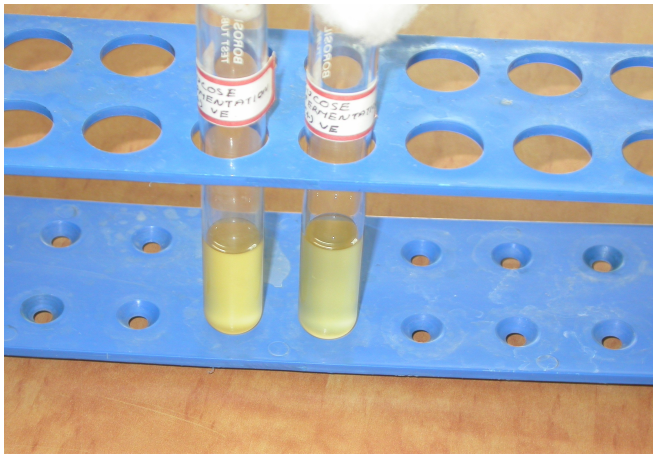
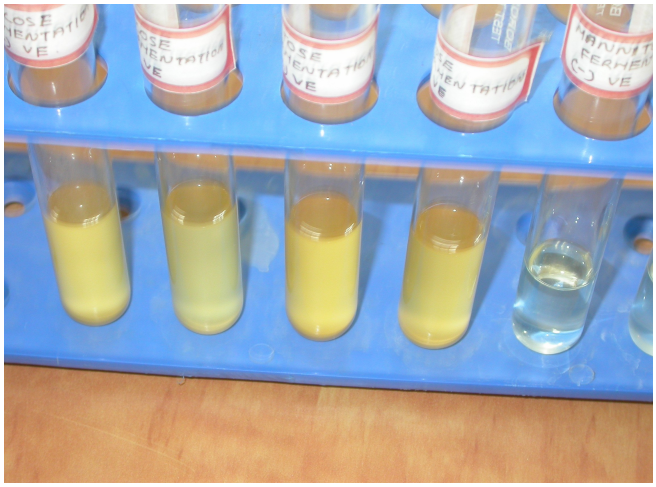


Figure No. 2: Biochemical test

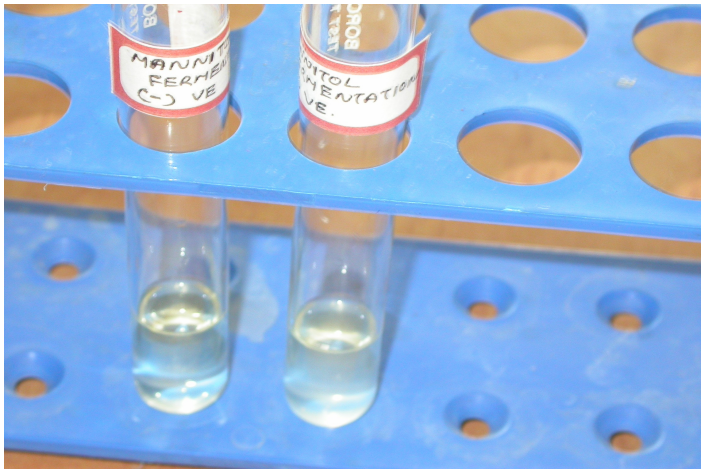
2a) Glucose fermentation test:



2b) Lactose fermentation test



2c) Mannitol fermentation test



2d) ST agar test:

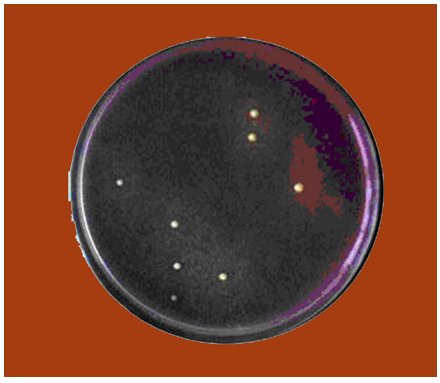


Figure No. 3: Gel Filtration Chromatography

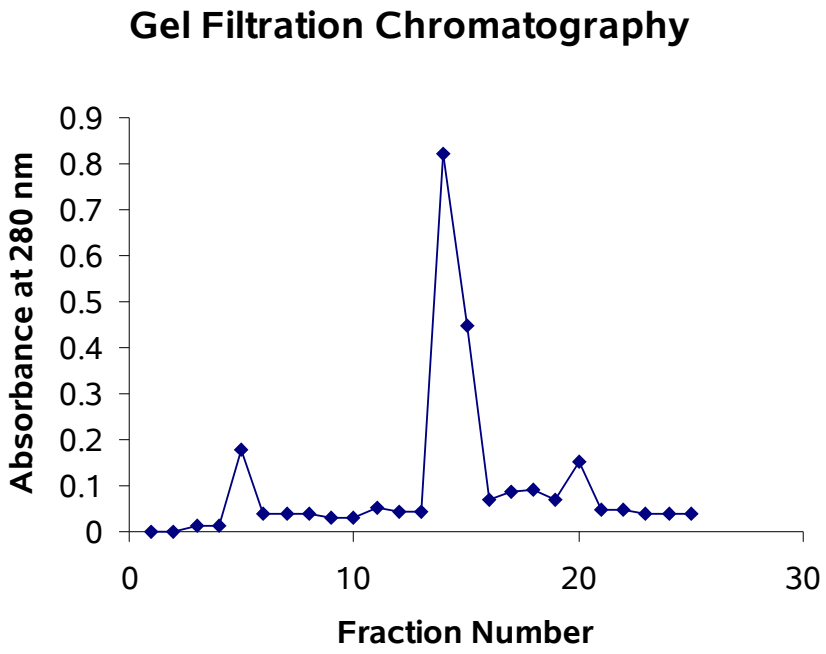


Figure No. 4: Standard Curve for BSA

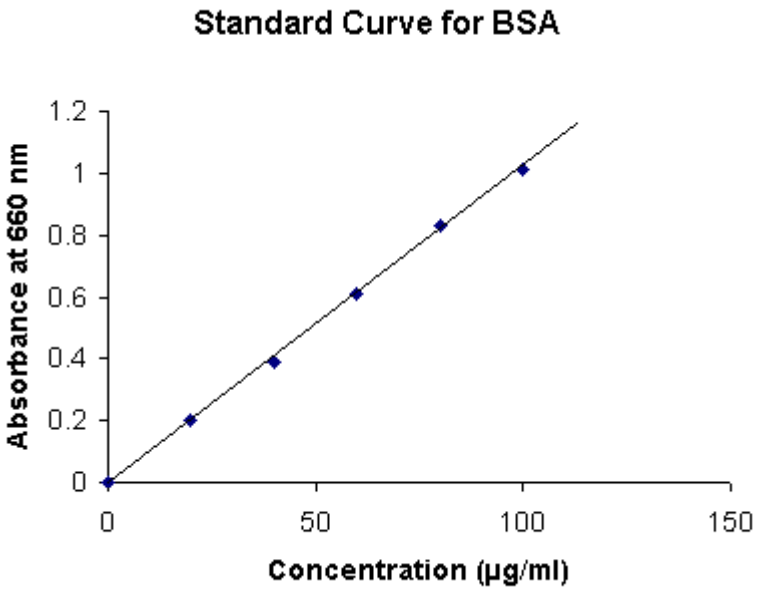


Figure No. 5: ONP Standard Curve

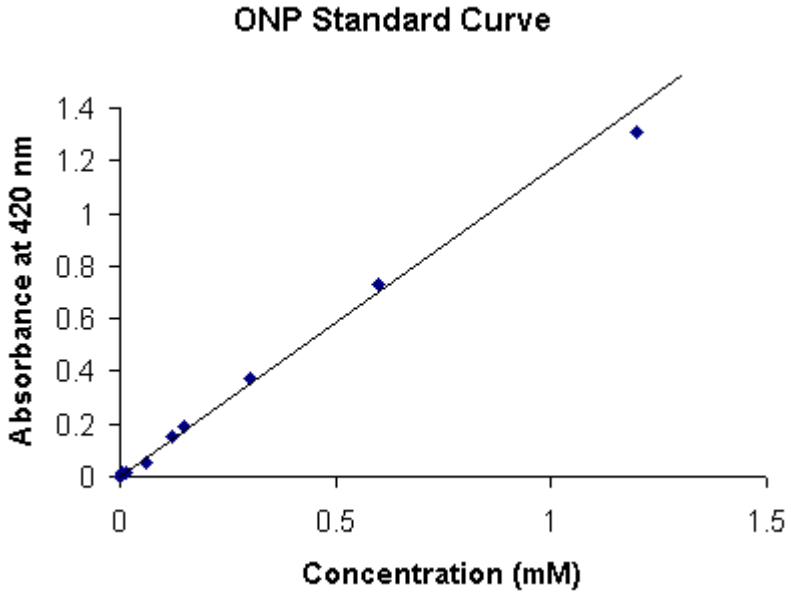


Figure No. 6: Standard Curve for Beta-Galactosidase

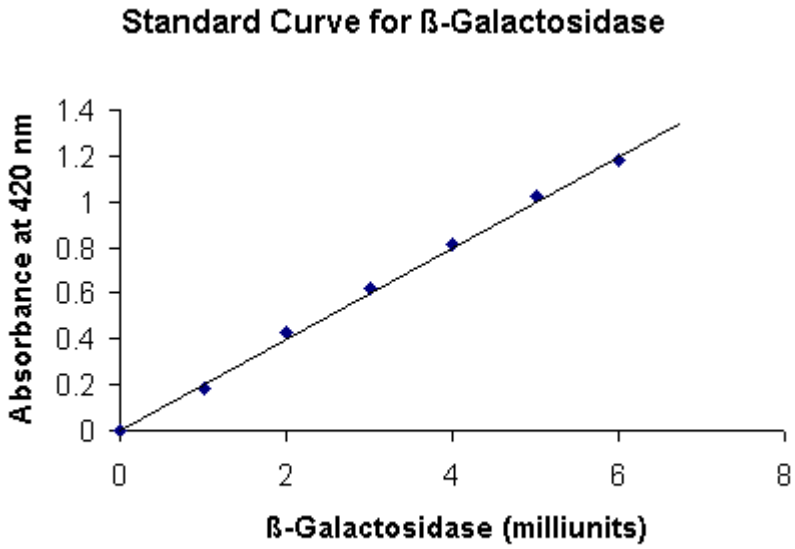
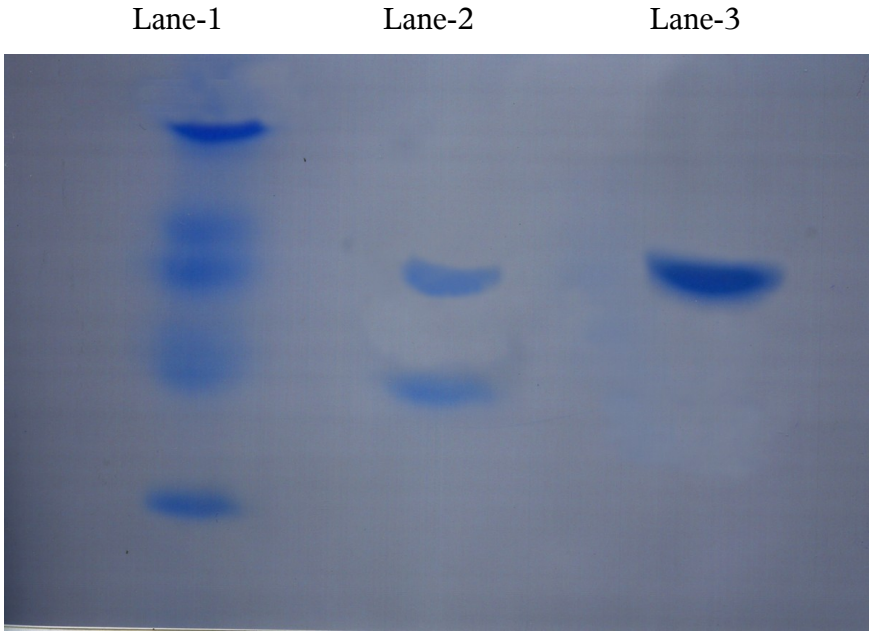


Figure No. 7: SDS-PAGE



Lane-1: Enzyme partially purified by ammonium sulphate precipitation.

Lane-2: Enzyme partially purified by gel-filtration chromatography.

Lane-3: Standard Beta-Galactosidase enzyme

For determining the purity of Beta-Galactosidase enzyme SDS-PAGE was performed. The proteins were separated according to their molecular weight.

Figure No. 8: ONPG confirmatory test for Beta-Galactosidase

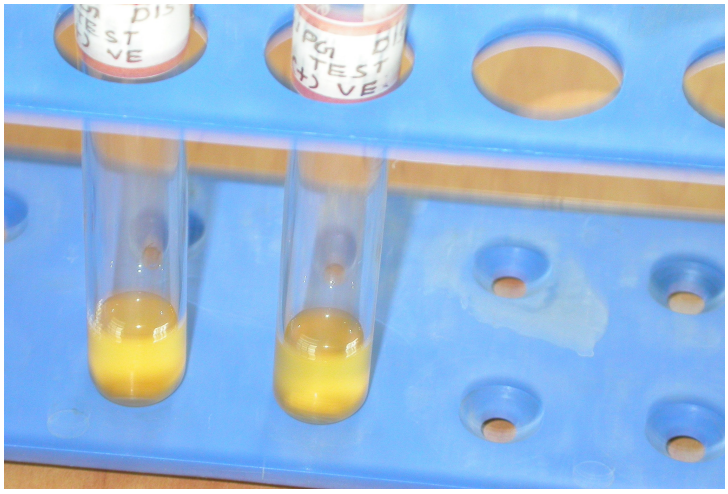


Figure No. 9: Optimization of Substrate Concentration

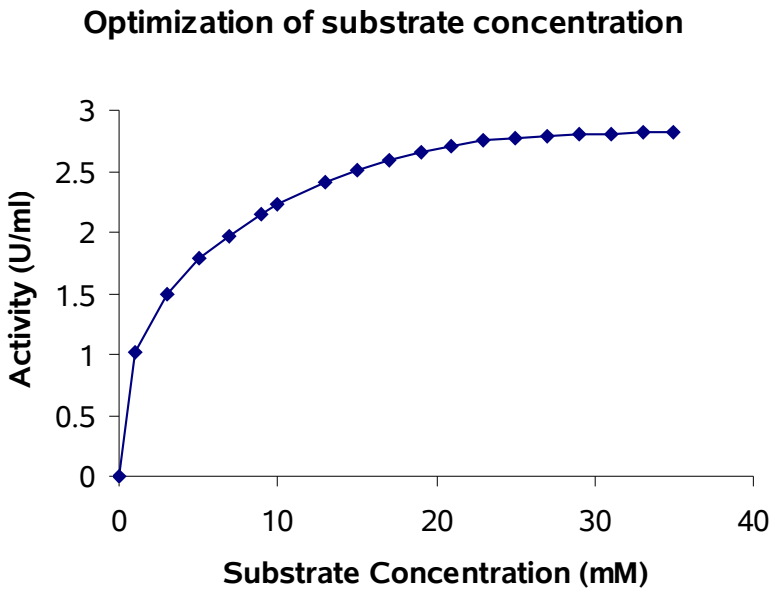


Figure No. 10: Determination of K_m and V_{max}

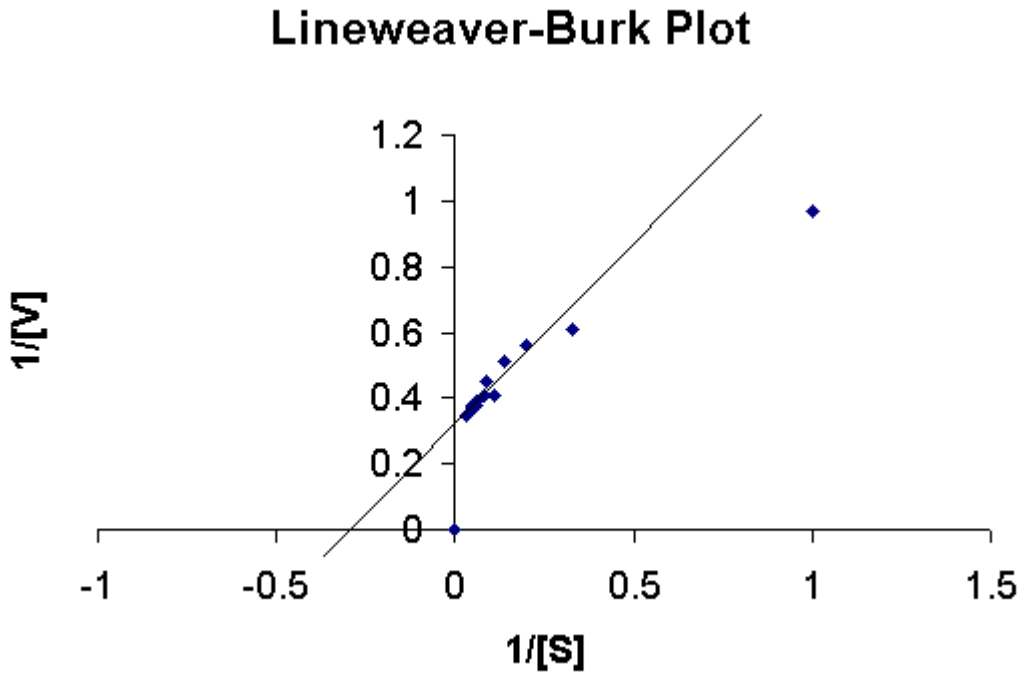


Figure No. 11: Effect of pH

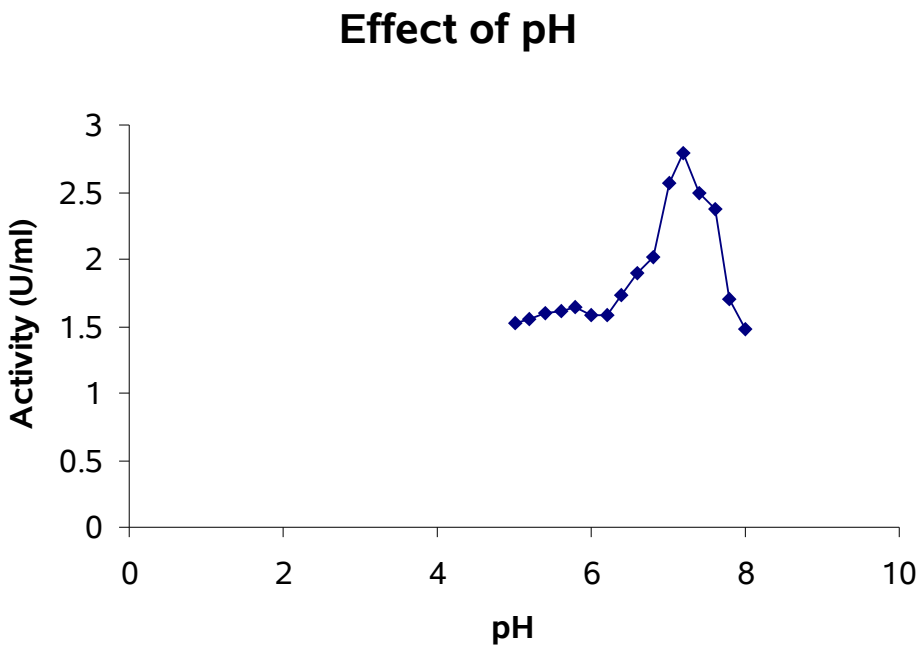
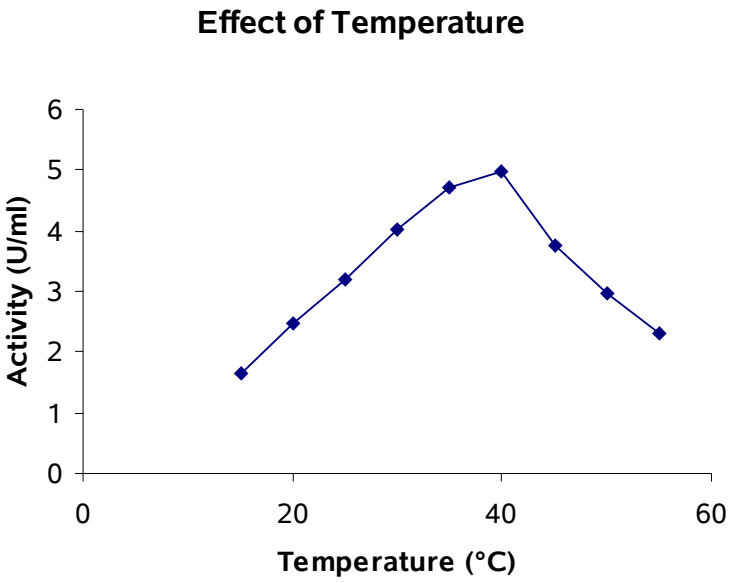


Figure No. 12: Effect of Temperature



8. SUMMARY AND CONCLUSION

Beta-Galactosidase was an intracellular enzyme isolated from *Streptococcus thermophilus* grown in whey. The Beta-Galactosidase from *Streptococcus thermophilus* exhibited twice the activity of enzyme from *Saccharomyces fragilis* being stable at 4 °C and having high heat stability.

Also whey utilization for the production of many valuable products has been extensively studied. One of the major obstacles to the whey utilization is lactose content, which causes crystallization at low temperatures, low sweetness, and poor digestibility when used as food. These problems can be solved if whey lactose is hydrolyzed to glucose and galactose.

Nine samples from commercial brand yoghurts namely, Amul (A1, A2, A3, A4, A5, A6, A7, A8, A9), Nilgiris (N1, N2, N3, N4, N5, N6, N7, N8, N9), and Nestle (NE1, NE2, NE3, NE4, NE5, NE6, NE7, NE8, NE9) were collected.

From Amul, the samples A1, A4, A5, and A6 showed clear white colonies and were isolated and subcultured in MRS broth for further studies. From Nilgiris, the samples N5 and N6 showed clear white colonies and were isolated and subcultured in MRS broth for further studies. From Nestle, the samples NE4 and NE5 showed clear white colonies and were isolated and subcultured in MRS broth for further studies.

Microscopical identification of the sample isolates were done. Five sample isolates A4, A5, A6, N6, and NE5 were identified as spherical or cocci shaped, gram-positive, and non-motile, having characteristics of *Streptococcus* species. Three sample isolates A1, N5, and NE4 were identified as rod shaped, gram-positive, and non-motile, having characteristics of *Lactobacillus* species. The five sample isolates A4, A5, A6, N6, and NE5, which are having characteristics of *Streptococcus* species were used for further studies.

Biochemical characterization was done for the sample isolates A4, A5, A6, N6, and NE5, which gave negative results for catalase test and mannitol fermentation test and positive results for glucose fermentation test, lactose fermentation test, and ST agar test. ST agar test was the identification test for *Streptococcus thermophilus*, so the five isolates A4, A5, A6, N6, and NE5 were subcultured in MRS broth and used for further studies.

Production of Beta-Galactosidase enzyme were done for the five isolates A4, A5, A6, N6, and NE5, examined synthesized beta-galactosidase with yields ranging from 5.07 to 8.36 U/ml. But the sample isolate A5 was selected for further studies because of high productivity (7.76 U/ml).

Partial purification of Beta-Galactosidase were done by Ammonium sulphate precipitation method (70%), dialysis, and gel filtration chromatography on Sephadex G-100. The fractions 5, 14, 15, 20 gave high peaks. In these, peaks 14, 15 showed enzyme activity. So these fractions were pooled and used for further studies.

The protein estimation was performed by Lowry's method and the protein concentration for the sample isolate A5 was found to be 67 µg/ml.

Purification of Beta-Galactosidase enzyme was performed by SDS-PAGE. The comparison was done with standard Beta-Galactosidase, the partially purified Beta-Galactosidase bands was shown near to standard Beta-Galactosidase band.

The assay of Beta-Galactosidase was performed by constructing ONP standard curve. The enzyme activity for sample A5 was found to be 7.76 U/ml.

The units of enzyme for sample A5 was found to be 3.6 milliunits by constructing standard curve using standard Beta-Galactosidase enzyme for determination of enzyme activity.

The ONPG confirmatory test for Beta-Galactosidase was done. The disc changed to yellow colour, which indicated the presence of Beta-Galactosidase enzyme.

The optimization of substrate concentration was performed using ONPG as substrate. The optimum substrate concentration was found to be 24 mM. The active sites of enzyme were saturated with the substrate molecules and blocked further action.

From the Lineweaver-Burk plot, K_m and V_{max} values were found to be 3.05 mM and 2.8 U/ml respectively.

The effect of pH and temperature was performed using ONPG as substrate. The optimum pH and temperature were found to be 7.2 and 40 °C.

Finally, it can be concluded that Beta-Galactosidase enzyme is a major source of Streptococcus thermophilus and also whey is utilized, which could otherwise be an environmental pollutant. The results presented here indicate that *S. thermophilus* grown in whey may represent a good source for the production of commercial lactase.

The *S. thermophilus* strain contained plasmids, which can be used as a vector in recombinant DNA technology. Also Beta-Galactosidase enzyme is used as a reporter gene in recombinant DNA technology and immunology.

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APPENDIX

1. HYA agar medium

Ingredients	Composition
Agar	15 gm
Peptone	10 gm
Beef extract	1 gm
Lactose solution	10 ml
Galactose solution	10 ml
Glucose solution	10 ml

Lactose solution:

5 gm of lactose was weighed and distilled water was added to make 10 ml.

Galactose solution:

2.5 gm of galactose was weighed and distilled water was added to make 10 ml.

Glucose solution:

2.5 gm of glucose was weighed and distilled water was added to make 10 ml.

Procedure:

All components (except the 3 solutions) were added to distilled water and the volume was brought upto 970 ml, mixed, gently heated and brought to boiling. pH was adjusted to 6.8, autoclaved for 15 min at 121 °C and cooled to 45-50 °C. The three solutions were added, mixed and poured into petri plates.

2. MRS broth

Ingredients	Composition
Peptone	10 gm
Yeast extract	5 gm
D (+) Glucose	20 gm
Tween 80	1 gm
Ammonium citrate	2 gm
Sodium acetate.	5 gm
$MgSO_4.7H_2O$	0.1 gm
$MnSO_4.4H_2O$	0.05 gm
Dipotassium hydrogen phosphate	2 gm

Distilled Water	1000 ml

3. Lactose broth

Ingredients	**Composition**
Beef extract	3 gm
Peptone	5 gm
Lactose	5gm

4. Glucose broth

Ingredients	**Composition**
Peptone	5 gm
D-Glucose	5 gm
Disodium hydrogen phosphate	5gm

5. Mannitol broth

i). 3 gm peptone was dissolved in 850 ml of distilled water.

ii). 5 gm yeast extract was dissolved in the solution from step (i).

iii). 25 gm of Mannitol was dissolved in the solution from step (ii).

iv). The volume was brought to 1000 ml with distilled water, then autoclaved or filter sterilized.

6. Streptococcus thermophilus agar: The ingredients of *S. thermophilus* agar (ST agar; 10.0 g of tryptone, 10.0 g of sucrose, 5.0 g of yeast extract, and 2.0 g of K_2HP0_4) were dissolved in 1 L of distilled water. The pH was adjusted to 6.8 ± 0.1, and 6 ml of 0.5% bromocresol purple and 12 g of agar were added to the medium. The medium was sterilized at 121 °C for 15 min.